NACO

MANUAL ON LABORATORY DIAGNOSIS OF COMMON OPPORTUNISTIC INFECTIONS ASSOCIATED WITH HIV/AIDS

Ministry of Health and Family Welfare
National AIDS Control Organisation

MANUAL ON

LABORATORY DIAGNOSIS OF COMMON OPPORTUNISTIC INFECTIONS ASSOCIATED WITH HIV/AIDS

Editors

Dr. Usha.K. Baveja
Dr. J. Sokhey

Government of India

National Institute of Communicable Diseases,

(Directorate General of Health Services), 22–Sham Nath Marg, Delhi – 110054.

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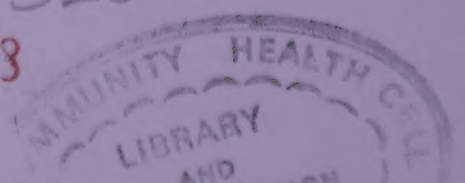
National AIDS Control Organisation

Ministry of Health & Family Welfare, Government of India, Nirman Bhavan, New Delhi-110011



सत्यमेव जयते

DR. S.P. AGARWAL
MS. (Surg.), M.Ch. (Neuro)
DIRECTOR GENERAL

GOVERNMENT OF INDIA
DIRECTORATE GENERAL OF HEALTH SERVICES
NIRMAN BHAVAN, NEW DELHI - 110011.
Tel. No. : 3018438, 3019063
Fax No. : 91-11-3017924

FOREWORD

HIV/AIDS is spreading at a rapid rate all over the world with an estimated 16,000 new infections every day. India is estimated to bear the largest load of HIV infected on account of the size of its population. It is estimated that about two-third cases in South and South East Asia are from India. More and more individuals infected with HIV are going to develop AIDS as there is no cure and or vaccine available for them. Antiretroviral drugs though, suppress the virus and prolong life are very expensive and have side effects. What can be done in this bleak scenario for HIV infected progressing to AIDS? The bulk of morbidity and mortality in HIV/AIDS is mostly due to opportunistic infections (OI). Prompt and accurate diagnosis and management of OIs will go a long way in making the life of AIDS patients comfortable and also prolonging life.

Most of the laboratory specialists involved in care of immunocompromised individuals may not be conversant with techniques involved in detection and identification of opportunistic micro-organisms. This manual titled "Laboratory diagnosis of opportunistic infections associated with HIV/AIDS" will help to develop expertise in this field.

The techniques described in the manual along with the photo-micrographs will help in early, accurate diagnosis and correct management of opportunistic infections in AIDS patients. The manual can also be used for training laboratory technologists in peripheral areas as some of the techniques described are simple and can be performed at PHC/CHC level also. This will thus alleviate the pain and suffering of people living with AIDS to a great extent.

I congratulate Dr. Usha K. Baveja, Consultant (Micro), NICD and her colleagues for bringing out such a useful document.

24.12.97

(DR. S.P. AGARWAL)

सत्यमेव जयते

J.V.R. PRASADA RAO
Addl. Secy. and Project Director
National AIDS Control Organisation
Tel: 3017706 Fax : 3014252

GOVERNMENT OF INDIA
MINISTRY OF HEALTH & FAMILY WELFARE
344 'A', Nirman Bhavan, New Delhi - 110001

MESSAGE

Opportunistic infections are significant contributors of morbidity amongst HIV infected individuals. Many of these infections are preventable and successfully treatable provided an accurate diagnosis is made. HIV/AIDS patients with opportunistic and other endemic infections may not have the typical symptoms pathognomic of a particular infection. So, laboratory diagnosis becomes important in management of these patients. Accurate treatment will go a long way in alleviating the pain and sufferings of HIV infected with opportunistic infections.

Many of the specialists working with HIV/AIDS patients may not be conversant with some of these infections as they rarely afflicted human beings before the era of HIV.

NICD has taken the initiative to prepare a manual of "Laboratory Diagnosis of Common Opportunistic Infections". The manual will help the laboratory specialists and all those working with HIV/AIDS patients in diagnosis and management of infections associated with HIV/AIDS.

I commend all those who have contributed for the preparation of this manual.

(J.V.R. Prasada Rao)

सत्यमेव जयते

JOTNA SOKHEY
Director

National Institute of Communicable Diseases
Directorate General of Health Services
22 - Sham Nath Marg, Delhi - 110054.

PREFACE

AIDS patients suffer from opportunistic infections on account of their compromised immune status. Opportunistic infections are defined as infections by organisms which do not normally causes disease, but may lead to severe, persistent and often life threatening illness in immunodeficient individuals.

Specific treatment of the opportunistic infections can improve the quality and prolong the life of the patients. Laboratory support is essential to determine diagnosis. Many of the laboratory tests can be performed at district level. It is, however, important that the tests are uniform and standardized and that adequate bio-safety precautions are observed at all times in the laboratories.

The manual on laboratory diagnosis of opportunistic infections should help in improving access to essential laboratory services in the country and in improving standards of laboratory practices in India. The manual would also be useful in training programmes for laboratory personnel and others involved in the care of HIV/AIDS patients.

I thank the national experts who have contributed chapters for the manual for their deep and personal involvement in this exercise. Dr. Usha K. Baveja, Head of the AIDS and HIV Division of the Institute has worked untiringly and it is entirely due to her sincere efforts that this document could be finalized within a short period of time.

Jotna Sokhey

(JOTNA SOKHEY)

सत्यमेव जयते

DR. (MRS.) USHA K. BAVEJA
Consultant (Micro) & Head Division of AIDS
National Institute of Communicable Diseases

GOVERNMENT OF INDIA
Directorate General of Health Services
22 - Sham Nath Marg, Delhi - 110054.

ACKNOWLEDGEMENTS

An early and accurate diagnosis of infections associated with HIV/AIDS is beneficial to both the patient as well as the physician in that it points to the aetiologic agent of infection and thus limits extensive further evaluation. It also helps in early accurate treatment and institution of prophylaxis (chemoprophylaxis) wherever indicated and relevant e.g. cases of Pneumocystis carinii pneumonia.

Most of the opportunistic infections being reported in AIDS patients are those which do not normally occur in human beings e.g. Pneumocystis carinii pneumonia, parasitic diarrhoeas due to coccidian parasites and toxoplasmosis etc. Some of the infections are those which were controlled and have re-emerged in a big way, for example tuberculosis. There are other infections which are endemic in the country e.g. leishmaniasis and malaria and these may accelerate the course of HIV disease by adding on to the immunosuppression in endemic areas. An acute need was felt to prepare a manual which will give a little background information about these infections with methods which are used to identify the micro-organisms causing these infections. The same has now been produced

I express my sincere thanks to Dr. S.P. Aggarwal, Director General of Health Services for technical guidance and for writing the foreword for the manual.

I express my sincere gratitude to Shri J.V.R. Prasada Rao, Additional Secretary and Project Director, National AIDS Control Organisation, Ministry of Health & Family Welfare for constant encouragement and support.

I thank Dr. Jotna Sokhey, Director, National Institute of Communicable Diseases for supporting this proposal and for her encouragement throughout.

I am most grateful to each and every technical expert for contributing various chapters inspite of their busy schedules. I especially thank Prof. P. Bhalla and Prof S. Broor for their contributions inspite of some mishaps in their families.

I gratefully acknowledge the financial assistance provided by WHO for printing this manual.

Finally, I express my thanks and appreciation for the way Mr. Mukesh Kumar worked on, even during Government holidays to type and set the manual.

UKBaveja
Dec. 1999

Dr. (Mrs.) Usha K. Baveja
Consultant (Micro) & Head
Division of AIDS, NICD, Delhi.

LIST OF CONTRIBUTORS

1. **Dr. Usha K. Baveja,** Consultant (Micro) & Head, Division of AIDS, National Institute of Communicable Diseases, 22 – Sham Nath Marg, Delhi.

2. **Dr. P.L. Joshi,** Joint Director, (Technical), National AIDS Control Organisation, Ministry of Health & Family Welfare, Nirman Bhavan, New Delhi.

3. **Dr. S.N. Mishra,** Consultant (WHO), National AIDS Control Organiaton, Ministry of Health & Family Welfare, Nirman Bhavan, New Delhi.

4. **Dr. D. Sengupta,** Former Consultant (Clinical), National AIDS Control Organisation, Ministry of Health & Family Welfare, Nirman Bhavan, New Delhi.

5. **Dr. D. Chattopadhya,** Joint Director, Division of AIDS, National Institute of Communicable Diseases, 22 – Sham Nath Marg, Delhi.

6. **Dr. Shobha Broor,** Professor, Department of Microbiology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi.

7. **Dr. P. Bhalla,** Professor, Department of Microbiology, Maulana Azad Medical College, New Delhi.

8. **Dr. Suma Ray,** Department of Microbiology, All India Institute of Medical Sciences, New Delhi.

9. **Dr. C. Rajendran,** Joint Director, Division of Microbiology, National Institute of Communicable Diseases, 22– Sham Nath Marg, Delhi.

10. **Dr. Sunil Gupta,** Joint Director, Division of Microbiology, National Institute of Communicable Diseases, 22– Sham Nath Marg, Delhi

11. **Dr. V. Mittal,** Joint Director, Division of Zoonosis, National Institute of Communicable Diseases, 22– Sham Nath Marg, Delhi.

12. **Dr. M. Bose,** Reader, Department of Microbiology, V. Patel Chest Institute, Delhi.

13. **Dr. Mandira Varma,** V. Patel Chest Institute, Delhi.

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SECTION –I : GENERAL

Chapter –1

OPPORTUNISTIC INFECTIONS IN HIV/AIDS PATIENTS : AN OVERVIEW

The first case of AIDS was reported in India in 1986. The case definition for AIDS in India has been modified twice since then and according to the new case definition a total of 8491 cases have been reported to the National AIDS Control Organisation till the end of September, 1999. The majority of these cases have been reported from Maharashtra (3379), Tamilnadu (3236) and Manipur (327). Though HIV infection has been reported from all the States and Union Territories, 5 States and Union Territories have not reported a single case of full–blown AIDS. They are Arunachal Pradesh, Andaman and Nicobar Islands, Dadra and Nagar Haveli, Lakshadweep and Tripura.

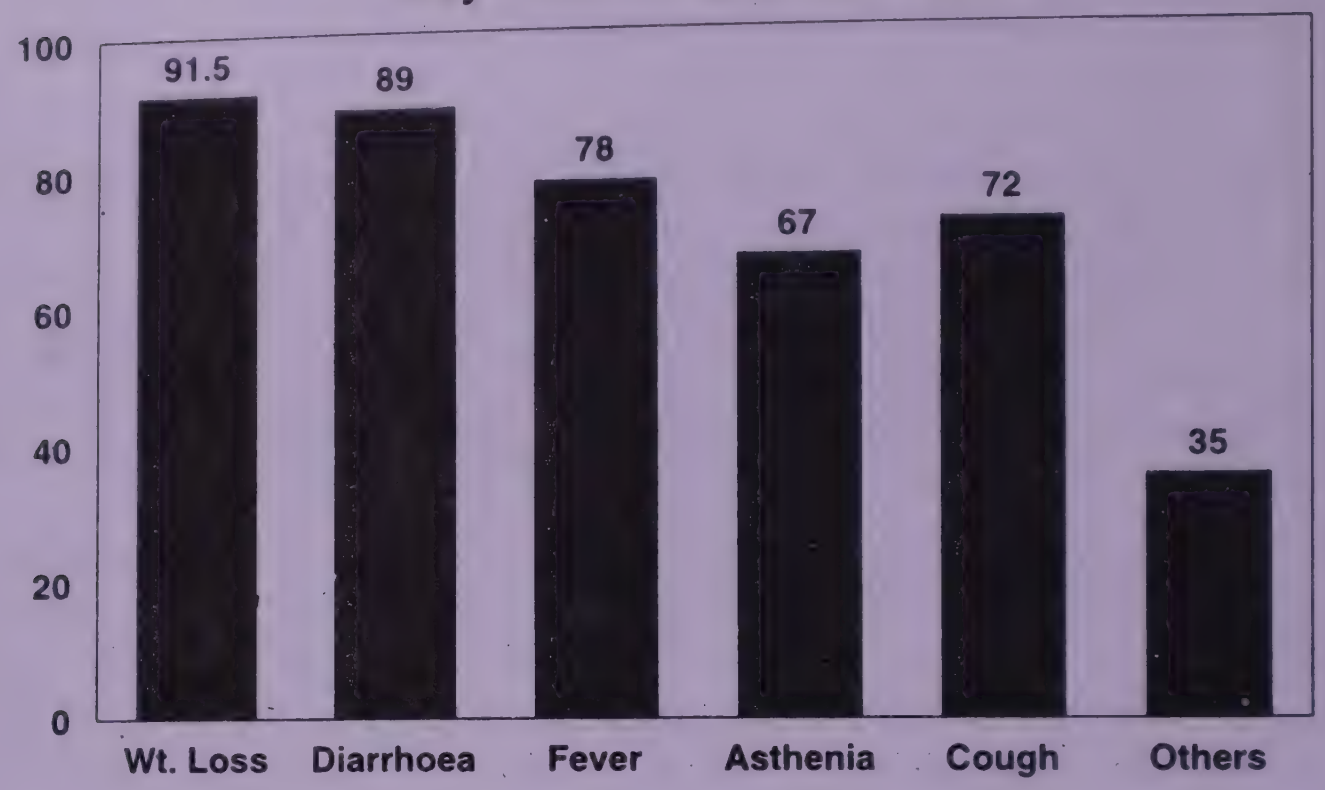
Most of the HIV/AIDS infections are in the age group of 15 to 49 years with heterosexual transmission being the predominant mode of HIV infection. About 79% of the cases are males and 21% are females.

The AIDS case definition encompasses a spectrum of infections and malignancies, labelled as opportunistic infections (OIs) and diseases. These infections are different in different geographical regions. In America and Europe, Pneumocystis carinii pneumonia, Cryptococcal meningitis, Cytomegalovirus infection and Toxoplasmosis are commonly seen among the AIDS patients. But in the developing countries of Africa and Asia, where tuberculosis and other tropical parasitic diseases are highly prevalent, the spectrum of OIs is rather different. In India, tuberculosis, both pulmonary and extrapulmonary were found to be the major opportunistic infections, accounting for 63% of the cases. Of these, 45% had extensive pulmonary tuberculosis, 4% had exclusive extrapulmonary tuberculosis and 14% had both pulmonary and extrapulmonary tuberculosis. The major extrapulmonary sites of tuberculosis were the lymph nodes, the liver and endometrium. Miliary tuberculosis and disseminated disease were seen in a few cases. This large number of tuberculosis infections coupled with HIV infection has thrown up the challenge of a dual epidemic in India.

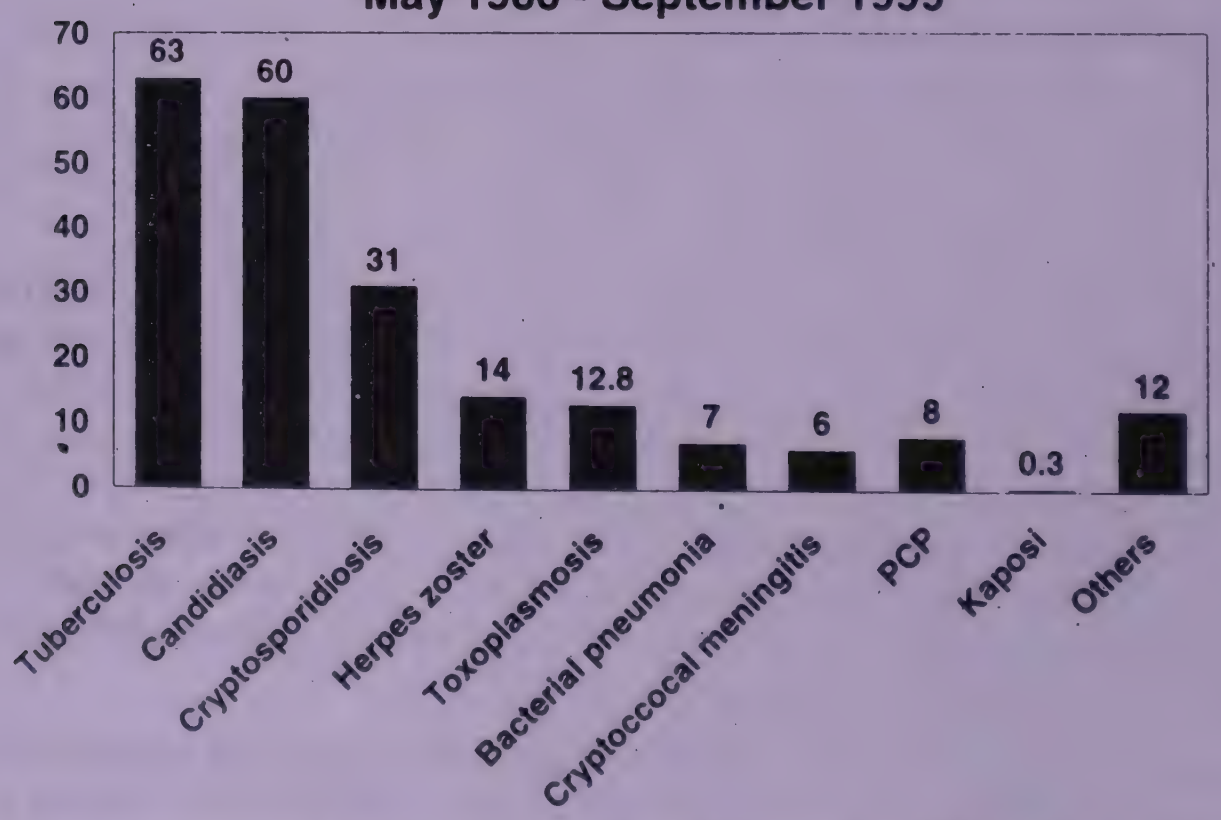
Candidiasis, caused by Candida albicans is the next common opportunistic infection. Oral and oesophageal candidiasis was seen in 60% of the AIDS cases. Other infections seen are Cryptosporidiosis causing diarrhoea, Herpes zoster, Toxoplasmosis, Pneumocystis carinii pneumonia and Cryptoccocal meningitis. Kaposi's sarcoma is a malignant condition seen frequently in AIDS patients. However, the number is low in India as compared to the west. Other infections seen, though in a small number of cases, were Cytomegalovirus, Herpes simplex, progressive generalised lymphadenopathy, oral hairy leukoplakia, non – Hodgkins lymphoma and lymphoid interstitial pneumonia. Recently, a rare fungal infection by Penicillium marneffei causing pneumonia has been reported from the HIV infected injecting drug user (IDU) in the North–Eastern part of the country.

Re–emerging infections like Coccidioidomycosis, Histoplasmosis, and disseminated Molluscum contagiosum have been reported in a very small percentage of AIDS cases. Chronic diarrhoea has been a presenting symptom in a majority of the AIDS patients. Though, in most of these cases, the pathogenic organism could not be identified, commonly reported infections have been due to Cryptosporidium, Isospora belli, Entamoeba histolytica and Giardia lamblia. A post mortem study done on AIDS patients in Mumbai found Cytomegalovirus as an important cause of intestinal infection.

Presenting signs and symptoms of AIDS cases in India (n=8491)
May 1986 - September 1999



Opportunistic infections in AIDS cases in India (n=8491)
May 1986 - September 1999



Diagnosis and surveillance of these opportunistic infections in AIDS will lead to early, accurate treatment and better management of these cases. HIV/AIDS may not be curable but most of the opportunistic infections can be effectively treated. Prophylaxis against some of these infections will not only prolong the life of an HIV infected individual but also improve the quality of life. So an early and accurate diagnosis of the aetiological agent is important.

Chapter –2

CLINICAL DIAGNOSIS OF HIV/AIDS

2.1 Introduction

AIDS, Acquired Immunodeficiency Syndrome, is not a single disease. It is the manifestation of a group of symptoms or disease processes which is why it is called a "syndrome"

So, diagnosis of AIDS only on clinical ground is difficult. It has to be suspected first on clinical symptomatology, on algorithmic approach and then has to be confirmed by serological tests. Primary Health Centres/Dispensaries have no X-ray or laboratory services available to diagnose HIV/AIDS related illnesses. The diagnosis of AIDS both in adults and children is difficult in the normal circumstances because the usual signs and symptoms are not pathognomic of HIV/AIDS and resemble many common illnesses.

Clinical signs and symptoms algorithm used for diagnosis of symptomatic HIV disease is helpful. One should always look for the epidemiological risk factors, cardinal findings, characteristic findings and associated findings to suspect AIDS.

2.2 Epidemiological risk factors

It is essential to take detailed history of the patient in relation to risk factors associated with acquiring HIV/AIDS infection.

Past or present history of risk behaviours

- Drug injecting
- Multiple homosexual contacts
- Recent history of genital ulcers/discharges
- History of transfusion of blood and blood products
- History of scarification or tattooing or tonn shaving

2.3 Cardinal findings

- Kaposi's sarcoma
- Pneumocystis carinii pneumonia
- Toxoplasma encephalitis
- Oesophageal candidiasis
- Cytomegalovirus retinitis

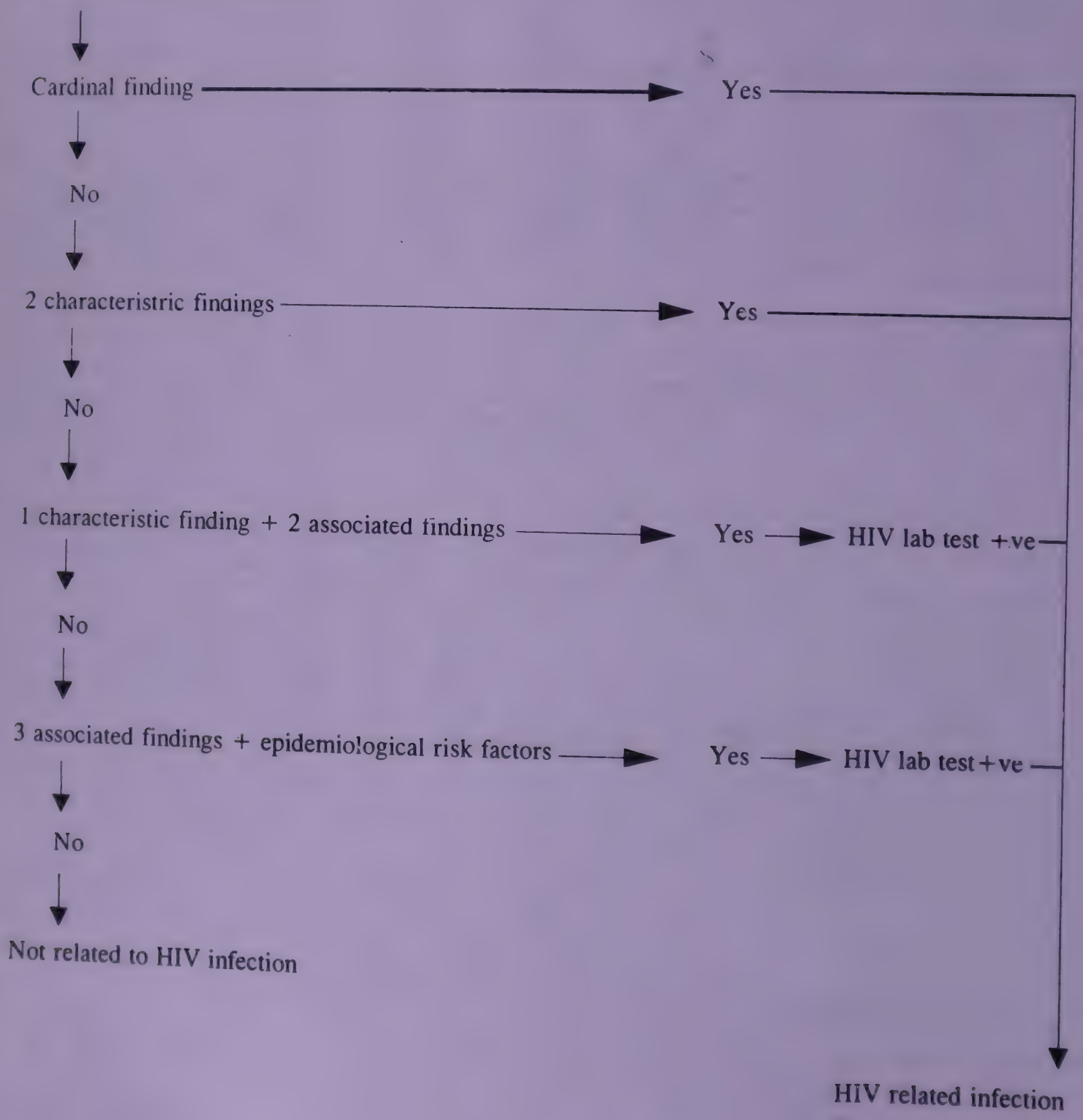
2.4 Characteristic findings

- Oral thrush
- Hairy leukoplakia
- Pulmonary tuberculosis
- Herpes zoster
- Severe prurigo
- Lymphoma

2.5 Associated findings

- Loss of weight of more than 10% of the basal body weight
- Prolonged fever for more than 1 month
- Diarrhoea for more than 1 month
- Neurological findings
- Persistent glandular lymphadenopathy (PGL)

Flow chart – 2.1 (Recognition of symptomatic HIV infection)



2.6 Case definition of AIDS (for persons above 12 years of age)

(i) Two positive tests for HIV infection (E/R/S) along with

(ii) Any one of the following criteria

(a) Significant weight loss (> 10% of body weight) within last one month/cachexia (not known to be due to a condition other than HIV infection) and chronic diarrhoea (intermittent or continous) > 1 month duration or prolonged fever (intermittent or continuous) > 1 month duration

(b) Tuberculosis : Extensive pulmonary tuberculosis, disseminated, miliary and extra pulmonary

(c) Neurological impairment preventing independent daily activities, not known to be due to the conditions unrelated to HIV infection (e.g. trauma)

(d) Candidiasis of the oesophagus (diagnosable by oral candidiasis with odynophagia)

(e) Clinically diagnosed life threatening or recurrent episodes of pneumonia, with or without aetiological confirmation.

(f) Kaposi's sarcoma

(g) Other conditions

- Cryptococcal meningitis
- Neuro toxoplasmosis
- CMV retinitis
- Penicillium marneffei infection
- Recurrent Herpes zoster infection (multi – dermatomal)
- Disseminated Molluscum contagiosum

Chapter –3

LABORATORY DIAGNOSIS OF HIV/AIDS AND NATIONAL HIV TESTING STRATEGIES

3.1 Introduction

Two distinct human immunodeficiency viruses, HIV-1 and HIV-2 are the aetiologic agents of AIDS. Phylogenetically, HIV-1 is divided into Group M [10 subtypes A-J] and Group O (9 subtypes) and Group N (new virus) and HIV-2 into 5 subtypes (A to E). In Thailand, India and sub-Saharan Africa, in 90% of HIV-1 infections are acquired through heterosexual transmission in contrast with 10% in the U.S. and Western Europe. Subtypes A, C and D predominate in Africa, subtypes E and B are commonly found in Thailand and C is the main subtype in India; whereas, subtype B predominates in the U.S. and Western Europe. As of the end of 1998, the World Health Organization estimates that a total of 33.4 million people are infected with HIV resulting in ~ 4.5 million AIDS cases worldwide.

In 50-93% of cases primary HIV infection is symptomatic with a variety of symptoms ranging from influenza-like or mononucleosis-like illness to more severe neurological symptoms which can persist from a few days to as long as two months; longer acute clinical illness is associated with rapid progression to AIDS.

Laboratory diagnosis is the only method for determining HIV status of such an individual. A number of tests and diagnostic kits are available to assess the HIV status of individuals. Serological tests are most commonly performed. The choice of test protocol depends upon :-

(i) Objective of HIV testing

(ii) Sensitivity and specificity of the test used

(iii) The prevalence of HIV infection among the population

(iv) Resources available

(v) Appropriateness to the strategy of testing

(vi) Infrastructural facilities available

Objective of HIV testing

(i) Blood and blood products safety. This is determined by mandatory screening of all donated blood

(ii) Donors of sperms, organs and tissues are tested for HIV to prevent HIV transmission to the recipient

(iii) Diagnosis of HIV infection in clinically suspected individuals

(iv) Voluntary testing : high risk groups after counselling

(v) Sentinel surveillance to monitor epidemiological trends

(vi) Research

Sensitivity

It is the accuracy with which a test can confirm the presence of an infection. Tests with high

sensitivity show few false negatives and are meant to be used to screen blood prior to transfusion and ensure blood safety.

Specificity

It is the accuracy with which a test can confirm the absence of an infection. Tests with high specificity show few false positives and are to be preferred for the diagnosis of HIV infection in an individual.

Prevalence of HIV infection

The probability that a test will accurately determine the true infection status of a person being tested varies with the prevalence of HIV infection in the population from which the person comes. The higher the prevalence, greater is the probability that a person testing positive is truly infected i.e. greater is the positive predictive value (PPV) of the test. The likelihood that a person showing a negative result is truly uninfected the negative predictive value (NPV) decreases as the prevalence of HIV infection among the general population increases.

3.2 Laboratory investigations for HIV infection

HIV infection can be detected in the laboratory either by detection of antibodies to HIV, or by detection of the virus, its antigen and its DNA. Detection of specific antigens, viral nucleic acid, isolation/culture of virus are all confirmatory tests in that the presence of the virus is detected. But they are risky because of the danger of infection to laboratory workers, are very laborious and difficult to perform, require skilled expertise and hence are to be done only in laboratories specified for research purposes.

The indirect predictors of HIV infection (CD4 cell count, $ß_2$ microglobulin etc.) are monitors of immunity status of patients and are to be done at routine intervals to monitor the progression of disease.

3.2.1 The specimens which can be utilized to detect HIV infection are

(i) Antibody detection

- Blood/serum/plasma

5 ml. of blood may be collected in clean, screw capped plain vial for ELISA and for the supplemental tests. Saliva and urine have been used to detect antibodies to HIV but the results have not been found to be satisfactory.

(ii) Antigen detection

- Serum/plasma
- Cerebrospinal fluid
- Cell culture supernatant (i.e. the tissue culture fluid)

(iii) Virus isolation and detection of viral nucleic acid

From HIV infected tissues. It can be successfully isolated from blood (PBMN cells), semen, vaginal/cervical specimen, tissue, CSF, and plasma. It is less successful on other body fluids like saliva, urine, breast milk, tears and amniotic fluid. Virus isolation is done for research purposes only.

3.2.2 Detection of specific antibodies

This is done by performing initial screening tests, which if positive, are followed up by supplemental tests to confirm the diagnosis.

Screening tests

ELISA : (Enzyme Linked Immunosorbent Assay)

It is the most commonly performed test to detect HIV antibodies.

There are various kinds of ELISA based on the principle of test :

- Indirect ELISA
- Competitive ELISA
- Antigen sandwich ELISA
- Antigen and antibody capture ELISA

ELISA is also classified on the basis of the antigens utilized into :

1st generation : Infected cell lysate is used as the antigen.

2nd generation : Glycopeptides (recombinant antigens) are used as the antigen.

3rd generation : Synthetic peptides are used as the antigen.

ELISA takes upto three hours to yieid results. It has a major advantage of being economical although rapid tests give result within minutes these are far more expensive. Commercial kits are available for ELISA and rapid tests. The recommended first line tests by NACO are ELISA. Rapid tests include:

(i) Dot blot assays

(ii) Particle agglutination (gelatine, RBC, latex, microbeads)

(iii) HIV spot and comb tests etc.

(iv) Fluorometric microparticle technologies

Simple tests are also based on EIA principle but take a little longer time, (> ½hr.) compared to rapid tests.

Tests which detect antibody to both HIV 1 and 2 are to be employed

When a serum sample tests reactive once by a system of ELISA/Rapid/Simple (E/R/S) test, the test is to be repeated immediately by a different system in order to confirm the diagnosis. If it tests reactive a second time, the sample is to be taken up for supplemental tests to confirm the diagnosis. Supplemental tests may be E/R/S, WB, IFA, RIPA etc

Supplemental tests

- Second and third ELISA/Rapid/Simple
- Western blot
- Indirect Immunofluorescence
- Radio Immuno Precipitation Assay

WB/IFA/IB. employed in problem cases and for research

W B, IFA etc. are expensive, time consuming and require expertise to perform. They are to be done to confirm the diagnosis on samples which give discordant results in E/R/S

3.3 Causes of false positive and false negative results

There are many conditions which may give rise to biological false positive or false negative ELISA results. False positive ELISA results have been reported in cases of haematologic malignant disorders, DNA viral infections, autoimmune disorders, multiple myeloma, primary biliary cirrhosis, alcoholic hepatitis, chronic renal failure, positive RPR test and false negative results have been reported in cases of window period prior to seroconversion, immunosuppressive therapy, malignant disorders, B-cell dysfunction and bone marrow transplantation.

3.4 Strategies of HIV testing

Because of the enormous risk involved in transmission of HIV through blood, safety of blood products is of paramount importance. Since the PPV is low in populations with low HIV prevalence, WHO/GOI have evolved strategies to detect HIV infection in different population groups and to fulfil different objectives. The various strategies, so designated, involve the use of categories of tests in various permutations and combinations.

1. ELISA/Simple/Rapid tests:

   used in strategy I, II & III

2. Supplemental tests : E/R/S and Western blot.

3.4.1 Strategy I

Serum is subjected once to E/R/S for HIV If negative, the serum is to be considered free of HIV and if positive, the sample is taken as HIV infected for all practical purposes. This strategy is used for ensuring donation safety (Blood, Organ, Tissues, Sperms etc.). Donor is not informed about the result. Unit of blood testing positive is destroyed as per guidelines

3.4.2 Strategy II

A serum sample is considered negative for HIV if the first ELISA test reports it so, but if reactive, it is subjected to a second ELISA which utilizes a system different from the first one. It is reported reactive only if the second ELISA confirms the report of the first. This strategy is used to diagnose HIV in individuals with AIDS indicator symptoms (Chapter-2) and for sentinel surveillance (anomymous, unlinked testing). Two different kits with different antigen system or different principle of test are required to follow this strategy.

3.4.3 Strategy III

It is similar to strategy II, with the added confirmation of a third reactive E/R/S test being required for a sample to be reported HIV positive. The test to be utilized for the first ELISA is one with the highest sensitivity (high number of false positives) and for the second and third ELISAs, tests with the highest specificity are to be used. This strategy is used for diagnosis of HIV infection in asymptomatic individuals. Counselling and informed consent are a must in these cases. Three different kits with different antigen system and/or different principle of test are required to follow this strategy.

Strategy II & III are to be used for diagnosis of HIV infection. E2 and E3 ought to be tests with the highest Positive Predictive Value (PPV) possible to eliminate any chances of false positive results.

Antibodies to HIV-1 are most commonly and reliably detected by EIA and confirmed by IB/WB. Antibody testing by EIA remains the standard method for screening potential blood donors;

simultaneous testing for p24 antigenemia is superfluous because of low sensitivity and expense. Use of improved, third generation serological assays demonstrates that seroconversion typically occurs 3–12 weeks post-infection, although significant delay can occur in some individuals; p24 antigen positivity and/or PCR detection of proviral DNA further narrows the window period following primary infection.

In diagnosing HIV-1 infection, the specificity of EIA is > 99% when properly performed and the sensitivity is >98%. In low-risk populations, the false-positive rate of combined EIA and WB/IB testing is estimated to be <1 in 100,000. Highly sensitive and specific agglutination and EIA methods for detection of type-specific antibodies to HIV-2 also are available.

3.5 Detection of p24 antigen

Detection of p24 viral antigen is expensive. The sensitivity of the test is also limited. Though, a positive test confirms HIV infection, a negative test does not rule out HIV infection. It is undertaken in the following situations :

(i) To detect infection in the newborn

(ii) To resolve equivocal Western blot results

(iii) To detect infection during early window phase

(iv) To diagnose CNS disease

(v) Late stage of disease (immune collapse)

(vi) For research

(vii) To monitor response to anti-retroviral therapy

EIA for HIV-1 antigen detects primarily uncomplexed p24 antigen, in serum, plasma, CSF or cell culture. It indicates active infection, allows diagnosis before seroconversion, can predict prognosis and is useful for monitoring response to therapy. Disadvantages of antigen detection assays include: poor sensitivity (only 69% in patients with AIDS and low in neonates <1 month old); detection is not possible in patients with high titers of p24 antibody (which complexes with the antigen); and failure to detect HIV-2 antigen.

3.6 Polymerase chain reaction (PCR)

PCR can detect proviral DNA during window period, can differentiate latent HIV infection from active viral transcription and can quantitate the copy number of HIV DNA when used with external standards (e.g. viral load assays). PCR can successfully differentiate between HIV-1 and HIV-2 infections. Proviral DNA can be detected in peripheral blood mononuclear cells before seroconversion. Limitation to the diagnostic use of PCR are rare false-negatives, some of which can be avoided by the use of multiple primer pairs and primers from conserved regions of the genome and false-positives due to cross-contamination of the PCR reaction mixture.

HIV-1 can be detected by PCR in the CSF of HIV-infected patients independently of disease stage; spread of HIV-1 to the brain represents an early event during infection which occurs in most asymptomatic individuals. PCR can also be used to detect HIV infection in neonates borne to HIV infected mothers.

3.7 Virus culture

Virus culture is another method for identifying HIV infection. Positive culture rates of up to 98%

are reported in confirmed seropositive individuals. The culture method is, however, expensive, labour-intensive, can take weeks for complete results and potentially exposes laboratory workers to high concentrations of HIV. Virus culture is used for research (drug sensitivity, vaccine studies etc.).

3.8 Viral load assay

Quantitation of HIV RNA in plasma is useful for determining free viral load, assessing the efficacy of antiviral therapy and predicting progression and clinical outcome. Because baseline HIV viral load is predictive of survival at 10 years in patients with nearly identical CD4 counts (70% survival with low viral load versus > 70% mortality with high viral load), assessment of baseline viraemia prior to initiation of therapy is useful in patient management. RT-PCR has higher analytical sensitivity (100 copies/mL plasma) than branched-chain DNA detection (5,000-10,000 copies/mL plasma).

3.9 Surrogate markers

Prognostic factors for progression to AIDS requiring further study include elevated serum prolactin concentrations, decreased dehydroepiandrosterone concentrations, the presence of antibodies of HIV-1 virion infectivity factor protein, and elevated serum IgE concentrations. The role of cytokines and cytokine receptors and their potential prognostic value in HIV infection also require further investigation in addition to the other well known prognostic factors. Refer to HIV testing manual for details, available from NACO, G.O.I.

Indirect predictors of HIV infection

i) Decreased CD4 cells

ii) Increased B2 microglobulin

iii) Increased serum neopterin

iv) Increased IL 2 receptors

v) Indicator diseases for AIDS

3.10 National guidelines for HIV testing

Need of HIV testing

The early detection of HIV infection differs from similar detection of most other infectious diseases on account of the following reasons : -

(i) Due to prolonged asymptomatic stage of infection, one remains fully active and demands an appropriate intervention which maintains the lifestyle and dignity of the individual.

(ii) HIV infection is believed to be invariably fatal irrespective of the best possible treatment.

(iii) HIV infection and AIDS are still associated with high degree of discrimination and stigmatisation.

(iv) The implications of a positive test go well beyond those related to physical and mental health of the individual being tested.

Thus, any HIV testing that is done has any of the four following objectives :

(i) To monitor the trends of HIV infection in a population or subgroup for facilitation of intervention (surveillance : unlinked and anonymous).

(ii) To test donated blood or donors of organs or tissues for ensuring safety of the recipients (transfusion safety and donation safety).

(iii) To identify an individual with HIV infection for diagnosis (with AIDS indicator diseases) or voluntary testing purposes (asymptomatic or AIDS cases).

(iv) Research.

HIV testing strategy

Often the separate objectives mentioned above cannot be met by a single testing strategy. Different objectives require separate testing procedures and the choice of tests (discussed in detail above). Based on relevant consideration different procedures and strategies of testing are adopted.

The different procedures for testing are :

Unlinked anonymous testing

Such type of screening or testing is not directed to the individual, but has as its objective, the public health surveillane of HIV infection. It is an epidemiological method for measuring HIV prevalence in a selected population with the minimum of participation bias. Unlinked anonymous screening offers a distinct advantage over mandatory or voluntary testing. Unlinked anonymous testing involves use of blood already collected for other purposes; therefore, the effect of selection bias will remain though, minimal and will depend upon time, location and other details of blood collection.

Voluntary confidential testing

Testing is often done for diagnostic purposes. Here it is important that the issues related to **confidentiality** receive great attention. Since this method is based on voluntary HIV testing or testing for diagnosis of HIV/AIDS cases, it is imperative to respect the individual's need to maintain confidentiality. By maintaining confidentiality, it will not only instill faith in the individual about the health care system in the community but also encourage more and more people practicing risk behaviour to come forward for an HIV test.

Mandatory testing

When testing is done without the consent of the patient and data could be linked to identify the person it is called "mandatory testing". Mandatory testing is recommended only for screening donors of semen, organs or tissues in order to prevent transmission of HIV to the recipient of the biological products.

The choice of tests is also based on the different objectives of HIV testing. The tests that are adopted are the ELISA, Rapid or Simple, clubbed together as 'E/R/S'. One E/R/S denotes test done on one single antigen preparation; two is when all positive samples on first antigen test are repeated on a second antigen preparation and three is when this test is repeated for a third time using a different antigen system. For transfusion safety purposes one E/R/S is used; for surveillance and diagnosis of full blown AIDS cases two E/R/S are used and for asymptomatic individuals three E/R/S are used.

General principles of HIV testing

Testing policy in general should consider the following points :

(i) It should be part of the overall comprehensive and preventive program.

(ii) Testing should be technically sound and appropriate.

(iii) Test procedure must be appropriate to the field situation.

(iv) Testing procedure must be cost effective.

(v) Laboratory procedure must be monitored for ensuring quality.

HIV testing in health care settings

The fear and apprehension that exists among health care workers in managing HIV infected individuals and AIDS patients is largely due to the minimal risk that exists of HIV transmission due to a needle stick or other sharp injury. Thus the demand for mandatory HIV testing of patients admitted in hospitals or undergoing surgery etc. This demand is neither rational nor appropriate. A mandatory HIV test is no substitute for **Standard Work Precautions** that need to be adopted for every patient in a hospital or any other health care setting On the other hand testing without **explicit consent** of the patient has been proved to be counterproductive in the long run. In the control of the HIV epidemic such testing can drive the target people underground and make it more difficult for launching interventions.

The **National testing policy** reiterates the following :

- No individual should be made to undergo a mandatory testing for HIV
- No mandatory HIV testing should be imposed as a precondition for employment or for providing health care services and facilities.
- Any HIV testing must be accompanied by a pretest and post test counselling services.

3.11 References :

1. Retroviral testing : Essentials for Quality Control and Laboratory Diagnosis, Ed. Constantine, N.T. Callahan, J.D., Watts, D.M. CRC Press, London, 1992.
2. HIV testing policies and guidelines. W.H.O. 1994.
3. AIDS HIV : Reference Guide for Medical Professional. 4th ed. Ed. Fahey, John L, & Flemmig, D.S. CIRID, UCLA Williams & Wilkins, London, 1996.
4. AIDS Etiology Diagnosis Treatment & Prevention 4th Ed Ed. Curran, J. Essex, M & Fauci, A.S. Lippincott– Raven, 1997.
5. AIDS Therapy ed. Ralph Dalin, Henry Masur and Micheal S. Saag. Publishers. Churchil Livingstone, 1999.
6. HIV Testing Manual : Laboratory Diagnosis, Biosafety and Quality Control. NICD & NACO document 1999.

Chapter –4

IMMUNOPATHOGENESIS OF HIV/AIDS AND OPPORTUNISTIC INFECTIONS ENCOUNTERED DURING THE COURSE OF HIV DISEASE

4.1 Introduction

An individual encounters a wide variety of infectious agents during the lifetime. In the normal or immunocompetent individual the usual outcome of such encounter is mounting of an immune response leading to the elimination of the agent. Human Immunodeficiency Virus (HIV) infection is unique in the sense that not only the host is unable to eliminate the virus resulting in chronic or persistent infection but also there is a progressive destruction of the immune system. As a consequence, the HIV infected individual becomes immunocompromised and suffers from a variety of life threatening opprtunistic infections (OI) and malignancies.

4.2 Events following entry of the virus into the body

The events following entry of HIV differ marginally depending on the route of entry. In case of infection acquired through blood, the virus is carried mainly by blood dendritic cells to the lymph nodes. On the other hand, following mucosal exposure (e.g. of urogenital tract or rectum in case of sexual route of acquisition), the mucosal dendritic cells or Langerhans' cells function as carrier of the virus to the lymph node.

Stage of primary infection and its immunologic correlation

Lymph node is the organ where the virus multiplies rather than in blood and such replication may occur even in the absence of lymphadenopathy. Within the lymph node the CD4 cells, coming in contact with the carrier blood dendritic/Langerhan's cells become infected followed by intense viral replication and eventually a burst of viraemia. This phase correlates with the acute or primary HIV syndrome characterised by flu like symptoms. The most striking feature of this phase is marked lymphopaenia (compared to normal level), mainly due to depletion of CD4 cells. However, qualitative immunological assessment during this initial phase demonstrates intact cellular immunity (both CD4 and CD8 mediated) as supported by unimpaired proliferative response to mitogens and soluble antigens as well as humoral immunity in the form of neutralizing antibodies against the various epitopes of HIV.

In course of time within the germinal center of the lymph node the free virions as well as the cells carrying the virus are trapped by the follicular dendritic cells (FDC). This trapping is responsible for decline in the plasma viraemia and the onset of latent or chronic phase. Determination of viral load in lymphoid tissue at this stage shows number of cells positive for HIV DNA (demonstrable by Polymerase Chain Reaction) and levels of expression of virus to be 1 to 3 logs higher compared to blood.

Several other phenomena contribute to the fall in plasma viraemia e.g. generation of cytotoxic T Lymphocytes (CTL) against the viral proteins resulting in the killing of virus infected cells, formulation of neutralizing antibodies that form complexes with the viral antigen alongwith complement (C) and which are eventually cleared by the RE system and CD8 cells mediated suppression of virus replication through release of a soluble mediator.

In course of time, the lymphoid tissue shows signs of disruption of architecture, abnormal location and involution of germinal centers, increased vascularity and fibrosis. Further, there is a decrease in the ability of FDC, already in the process of depletion, to trap the virus resulting in spillage of the virus into the blood and re-emergence of viraemia in the advanced stage of the disease.

4.3 Cells of the immune system involved in the pathogenesis

Selective tropism of HIV

A subset of T lymphocyte characterised by the presence of a glycoprotein molecule on its surface termed CD4 as well as CD4 cells of monocyte lineage have been found to be the main targets of attack by HIV. In addition, epidermal and mucosal Langerhan's cells, by virtue of presence of CD4 molecule on the surface are also affected by HIV. Majority of the HIV strains are capable of replicating in both macrophages and CD4 lymphocytes although they are termed monocyte/macrophage tropic.

Viral determinant for virus and lymphocyte/macrophage interaction has been found to be between the gp120 subunit of HIV envelope protein, particulary the V3 loop region. On the side of the cells (i.e. CD4 lymphocytes and macrophages), although CD4 has been the primary receptor for both types of cells, separate co-receptors, belonging to the G protein family, are crucial for successful invasion. The co-receptor for T lymphocyte tropic virus is termed fusin or CXCR-4 while that for macrophage tropic virus is termed CCR-5.

4.3.1 T cell abnormality

Both CD4 and CD8 cells have been affected in HIV infection although they vary in terms of degree of involvement and relative contributions in immunopathogenesis.

CD4 cell abnormality

In HIV infection there is progressive depletion of CD4 cells leading to massive decline in counts. However, it is difficult to explain the defects in the CD4 cell mediated function solely on the basis of quantitative depletion, thereby pointing out to the added contribution of functional abnormalities.

CD4 cell depletion

Virological mechanism of CD4 cell depletion/disfunction

HIV can kill CD4 cell either (i) singly through accumulation of unintegrated viral DNA or inhibition of cellular protein synthesis or (ii) through formation of syncytia in which a single infected cell may form syncytia with hundreds of noninfected CD4 cells.

Nonvirological mechanisms of CD4 cell depletion

The following mechanisms are thought to be responsible for nonvirologic means of CD4 cell depletion.

Autoimmune phenomenon

Sharing of determinants of HLA class I/II molecules and HLA DR/DQ molecules with HIV envelope proteins have been demonstrated. Thus antibodies to HIV envelope proteins may cross react with these self antigens leading to loss of cells.

Anergy

It has been demonstrated that gp120 or gp120 anti gp120 complexes that bind to CD4 cells relay faulty messages to uninfected CD4 cells leading to anergy.

Superantigens

It is thought that HIV proteins (? doubtful) or other microbial proteins associated with multiple infections in HIV infected individuals are capable of binding nonspecifically at different sites on the â chain of TCR rather than the conventional â chain leading to massive stimulation, anergy or deletion of T cells.

Apoptosis

Apoptosis or programmed cell death can be considered as a physiologic phenomenon whereby the T cells reactive against the self antigens are eliminated. In HIV infection gp120 or gp120 - anti gp120 complexes are capable of passing a signal to CD4 cells which coupled by signals from other microorganisms lead to elevated degree of apoptosis.

Blind T cell homeostasis

It has been proposed that in the early stage of the disease following depletion of CD4 cells, body tries to maintain the quantitative pool of total T cells in a blind manner resulting in generation of CD4 and CD8 cells uniformly. The result is a relative decline of CD4 cells.

Immune depletion

Inability of the immune system to regenerate CD4 cells proportionate to the rapid and massive destruction as well as destruction of progenitor cells in thymus and bone marrow could be responsible for decline in CD4 cells count.

Functional abnormalities of CD4 cells

Virtually every function of cell is impaired in HIV infection some of which are mentioned below

Defective response to recall antigens in skin tests for delayed cutaneous hypersensitivity reactions

This defect is most conveniently assessed by testing a panel of skin test antigens like tetanus toxoid, diphtheria toxin, tuberculin, candidin, tripcophytin etc., for delayed cutaneous hypersensitivity reaction. Upon intradermal inoculation of the test antigen due to production of soluble mediators like macrophage activating factor (MAF), macrophage inhibitory factor (MIF) etc., by the Langerhan's cells on contact with CD4 cells leading to deficiency in attracting and holding of additional macrophages at the reaction site which is essential for the delayed cutaneous reaction. In case of HIV infection, due to depletion, inadequate number of CD4 cells are able to reach the local site hampering the delayed cutaneous reactivity.

Impaired in vitro proliferative response to soluble antigens

Impaired proliferative response of peripheral blood mononuclear cells to antigens, as well as to T cell (PHA) and B cell (PWM) mitogens in HIV infected individuals was initially thought to be either due to quantitative depletion of CD4 cells in case of T cells proliferative response or lack of adequate stimulatory signal from CD4 cells to the B cells in case of B cell proliferative response. However, later on it was found that in addition to such quantitative defects, both T and B cells have intrinsic functional defects.

Impaired production of IL-2

Interleukin-2, a lymphokine released from antigen stimulated CD4 cells is required for an effective immune response. It is esential for stimulation of B cells, Natural Killer cells, mononuclear

phagocytes and activated cytotoxic CD8 cells. Consequent to quantitative and qualitative depletion of CD4 cells in HIV infection the functions of all these cells are impaired as described subsequently.

Decreased y IFN production

Gamma interferon (yIFN), a cytokine released from CD4 cells is a potent activator of mononuclear phagocytes resulting in the release of reactive oxygen metabolites and nitric oxide ions which are instrumental in killing of intracellular pathogens like Toxoplasma gondii, Candida albicans and Pneumocystis carinii. In HIV infection due to impaired production of yIFN, there are frequent infections due to these agents. yIFN is also a strong inducer of class I and class II MHC antigens on the macrophages through which antigen presentation to T cell is mediated. In HIV infection, antigen presentation, including its own antigen is thought to be improper.

Decreased ability to help B cell for production of specific antibodies

Impaired signal for cytotoxic CD8 cells and NK cell mediated direct killing of virus infected cells.

These two functions are explained in the respective sections subsequently.

4.3.2 CD8 T cell abnormality

Many protective functions of CD8 cells are dependent on signals from CD4 cells. In the absence of such signals killing of virus infected cells through cytotoxic CD8 cells (direct as well as antibody dependent i.e. ADCC) as well as by NK cell is hampered resulting in increased incidence of viral infections like CMV, HSV etc. In addition, a phenotype subset of CD8 cells i.e. CD8+/CD38 - has been found to be characteristic in the individuals having slow course of the disease progression with stable CD4 cell count while the phenotype of CD8+/CD38 is characteristic of individuals showing rapid progression of disease.

4.3.3 B cell abnormalities

B cells are not normally infected by HIV. However HIV or its proteins like gp41 can activate B cells directly in a T-independent pathway. Secondly, EB virus which is frequent among HIV infected individuals can also activate B cells directly. These two potent activators of B cells, through a mechnanism defined as polyclonal activation, are responsible for hyper- gammaglobulinaemia, formation of circulating immune complexes and autoantibodies frequenly encountered in HIV infection. More importanly, functionwise the B cells are defective (poor) in response to exogeneous antigens or bacterial immunogens e.g. proteins and polysaccharide antigens due to inadequate help from CD4 cells through lymphokine mediators like IL-2 and IL-4 which is responsible for spectrum of bacterial infections e.g. Strept. pneumonae, H. influenzae etc.

4.3.4 Abnormalities of monocytes/macrophages

The various abnormalities demonstrable in monocytes include defective chemotaxis and oxidative burst responses, impaired secretion of IL-1, impaired function of antigen presentation, as well as Fc and C3 receptor mediated activity. These impairements of function cannot be explained only on the basis of infection by HIV since HIV has a relatively lower potential of cytopathogenicity for monocytes but rather are linked with unimpaired CD4 cell function as explained earlier.

Abnormalities of natural killer (NK) cells

NK cells representing the large granular lymphocytes that are characterised as a nonT nonB non monocyte cell, have physiologic function of direct killing of virus infected cells and tumour cells. In HIV infection NK cells show functional impairment responsible for invasion by opportunistic viral infections like CMV, HSV etc., as well as for a variety of tumours. The functional impairement of NK cell function may be linked with defective signal from CD4 cells.

4.4 Cytokine regulation and disease progression

Recently, considerable emphasis has been laid on role of cytokines in regulation of immunopathogenesis in HIV infection. The T helper (Th) cells have been charcterised into two subgroups i.e. Th1 and Th2 with differing pattern of cytokine profiles. Th1 pattern of cytokines are characterised by secretion of IL-2, IFN , TNFá and TNT-â. These Th1 specific ctyokines are associated with protective response. On the other hand the Th-2 specific cytokines i.e. IL-4, IL-5 and IL-10 are associated with disease progression. It has been postulated that so long there is a predonmiance of Th1 specific cytokine pattern, the virus remains dormant within the lymphoid cells characterised by asymptomatic phase. In the event of a swich over from Th1 to Th2 specific response, the virus transforms into a replicating form resulting in progressive lysis of lymphoid cells and progression into clinically active stage. It has also been suggested that Th1 specific cytokine response may be responsible for protection of many individuals from successful HIV infection after exposure to HIV even after entry of the virus since many HIV negative individuals with high risk behaviour show proliferative lymphocyte responses to several HIV proteins.

4.5 Opportunistic infections in HIV infected individuals in relation to various immunological stages

4.5.1 Initial infection : Acute seroconversion syndrome

Although this phase is characterised by marked CD4 lymphopaenia, the level of decline is not sufficient to invite any opportunistic infection except when coupled with qualitative defects rare cases of self limiting Pneumocystis carinii, oesophageal candidiasis or oral thrush may be encountered.

4.5.2 Early HIV disease

Early HIV disease is defined as the stage when the CD4 cell count is greater than 500 cells/ μl. Opportunistic infections are not common at this stage except occasional cases of pulmonary turberculosis, mycobacterium avium complex (MAC), histoplasmosis, herpes simplex labialis and oral hairy leukoplakia due to EB virus.

4.5.3 Intermediate stage disease

This stage of disease is defined by a CD4 count between 200 to 500 cell/il and is characterised to be asymptomatic in most individuals. However, bacterial sinusitis, bronchitis and pneumonia are more frequent. The causative organisms are those usually present in the respiratory tract such as Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and Mycoplasma pneumoniae. These organisms are not included in the AIDS defining illnesses.

4.5.4 Late stage diseases

Late stage of HIV disease is defined by a CD4 cell count between 50 to 200 cells/μl. The most common opportunistic infection to affect this group is Pneumocystis carinii pneumonia (PCP) so as to merit introduction of PCP prophylaxis in all patients at this stage. Patients at this stage are also at risk for acquiring Toxoplasma gondii infection, cryptosporidiosis, isósporiosis and oesophageal candidiasis.

4.5.5 Advanced HIV diseases

This stage is defined as a CD4 count less than 50 cells/ìm. The common opportunistic infection include MAC diseases, Cryptococcal meningitis, Cytomegalovirus retinitis, polyoma virus (responsible for progressive multifocal leukoencephalopathy), invasive aspergillosis, disseminated forms of histoplasmosis, coccidioidomycosis, bartonellosis and disseminated Penicillium marneffei infection. At this stage in addition to PCP prophylaxis, MAC prophylaxis is also recommended.

Stage of the disease	CD4 cell count	Prevalent opportunistic infection /diseases
Initial infection (Acute seroconversion syndrome)	much above 500 cells/μl	Rarely reported and self limiting variety of Pneumocystis carinii, esophageal candidiasis
Early HIV disease	> 500 cells//μl	Occassionaly reported : Pulmonary tuberculosis, MAC, histoplasmosis, Herpes simplex labialis, EB virus (hairly cell leukoplakia)
Intermediate stage	200–500 cells/μl	Strept. pneumoniae, H.influenzae, Moraxella ,catarrhalis, Mycoplasma pneumonae
Late stage	50–200 cells//μl	Pneumocystis carinii, Toxoplasma gondii, Cryptosporidia, Isospora, oesophageal candidiasis
Advanced stage	< 50 cells//μl	MAC disease, Cryptococcal meningitis, invasive forms of aspergillosis, histoplasmosis, coccidiodomycosis, bartonellosis, Penicillium marneffei infection, CMV retinitis, PML

4.6 Immunologic parameters as predictors of HIV diseases progression

Among the various immunological parameters assessed about their applicability as predictors of HIV disease progression, CD4 cell count, specially serial CD4 counts at specific intervals have been found to be most reliable. Clinicians routinely need serial assessment of CD4 cell count before taking any therapautic decision like initiation of antiretroviral therapy or PCP prophylaxis. The applicability of other parameters like ß–2 microglobulin, neopterin and p24 antigenaemia have not gained favour among clinicians. ß–2 microglobulin and neopterin have small dynamic range while p24 antigen is of limited value because large proportion of patients even with advanced disease will show negative p24 antigen test despite attempts to dissociate p24 – anti p24 complexes. Skin tests for DTH against the recalled antigens have also not been widely accepted. However, past several years have seen the remarkable advance of molecular techniques like RT–PCR, bDNA to quantitate viral-DNA which have found considerable applicability shadowing many age old immunological parameters for assessment of the disease stage or progression. Thus, the role of immunological parameters are now considered to be more of adjuncts than primary ones.

Chapter –5

STANDARD BIOSAFETY PRECAUTIONS

5.1 Introduction

Microbiologists and other laboratory technologists work in unique environment which may cause infectious disease to them if proper precautions are not taken. Since this manual deals with HIV/AIDS associated infections the biosafety precautions to be undertaken for HIV/AIDS (in fact apply to all infections transmitted through blood and body fluids) are given in details with some mention of biosafety level used in Microbiology Laboratories dealing with such infections. Preventive measures to be undertaken while dealing with Mycobacteria are described in details in the chapter on HIV/AIDS associated Mycobacterial infections.

5.2 Containment

This indicates methods, techniques, barriers and facilities etc. which protect the personnel as well as the environment (immediate and distant) from exposure to infectious agents.

Elements of containment

- Each personnel working in the laboratory should have adequate and correct knowledge of techniques, practices and equipments being used in the laboratory.
- Strictly adhere to standard microbiological practices and techniques.
- Should have training and knowledge about the infectious potential and hazard posed by the agent.
- Standard operative procedures for processing the specimens for various micro-organisms should be available with each personnel working in the laboratory.
- Each laboratory should also have a biosafety operation manual which will identify the hazards which may be encountered while working in the laboratory.
- All the above has to be supplemented with appropriate facility design, engineering features, safety equipment and management practices.

Barrier precautions

These are of two types. Primary barriers and secondary barriers.

Primary barriers for working in the OIs laboratory include biological safety cabinet class II (to prevent aerosol acquired infections e.g. tuberculosis) and personal protection such as gloves, coats, gowns, shoe covers, boots, face shield and safety glasses. These personal protective devices used in combination with class II biological safety cabinet are adequate with micro-organisms causing OIs in HIV/AIDS

Secondary barriers are to provide safe environment to personnel working in the laboratory as well as others working in the facility and the whole community in general. The laboratory design and the civil structure provided should be commensurate with the laboratory functions. The basic requirements for laboratory (space etc.) are given in the chapter on infrastructural requirements (Chapter–7).

Most of the opportunistic and other micro-organisms infecting HIV/AIDS patients are opportunistic or moderate risk agents. So, the laboratory engaged in this kind of diagnostic work should have equipments, facilities and practices commensurate with biosafety level-2 i.e. partial containment equipment i.e. biological safety cabinet class II. The biosafety precautions described in chapter on Mycobacterium suffice to process specimens for the HIV/AIDS associated infections. Access to laboratory is limited. All procedures are performed carefully so as to minimise aerosols.

5.3 Biosafety level - 1 (BSL-1)

Practices, equipment and facilities at this level are appropriate for working with micro-organisms that do not cause disease in healthy humans. Examples of such laboratories are secondary school, college teaching and municipal water testing laboratories etc.

The basic lay out of BSL-1 laboratory includes restricted entry and biohazard sign on the door, a hand washing sink near the door and adequate space for containers/bags as appropriate for waste materials (chemical, toxic, radioactive etc.). The work is done on the open bench. Top of the bench should be impervious to acid and easily cleanable.

The standard microbiological practices at BSL-1 include use of mechanical pipetting devices, adherence to the standard biosafety and barrier precautions (no eating, drinking and smoking in the laboratory; use of laboratory coat, eye shield and gloves and hand washing after each task).

5.4 Biosafety level - 2 (BSL-2)

The infrastructure at this level is adequate to carry out clinical, diagnostic and research work involving moderate risk micro-organisms. The access to laboratory is restricted. The activities can safely be conducted on the open bench provided the potential of micro-organism for producing aerosols is low. Any work which involves splatters or aerosols of infectious materials should be done in biological safety cabinet (BSC) and other containment devices. The examples of BSL-2 level micro-organisms include HIV, HBV, Toxoplasma gondii, Salmonella etc. transmissible by ingestion, exposure to mucous membrane or intradermal exposure. Government of India recommends HBV immunization of the laboratory workers handling blood, body fluid etc. at BSL-2.

The basic design of laboratory affords environment which maximises safety to the laboratory workers. The door to the laboratory is kept closed, waste materials generated in the laboratory must be segregated into colour coded containers and bags. Adequate space must be made available for this.

Standard microbiological practices of BSL-1 are effective at BSL-2 also with emphasis on mechanical pipetting devices, use of barrier precautions and attention to sharps. Do not break or bend needles. In most situations it is prudent to use single-use needles and syringes. Do not recap needles. Needles and syringes, butterfly needles and associated tubing, and similar devices should be discarded intact into a puncture and leak proof container. Other sharps items (such as broken glass) should not be handled by hand. Consider substituting plastic ware for glass laboratory items.

Other special practices include decontaminating work surfaces after completing the work with the infectious materials and reporting all spills and accidents. An incident log book is a useful means for recording events that have gone wrong; it is important to document these events, not for punitive action, but to be able to better understand what happened with an eye to preventing similar events in the future.

At BSL-2, all work that might create aerosols of infectious materials should be done in containment. The most common device is the biological safety cabinet, and the most common cabinet in use is referred to as a class-II, type-ABSC. Room air is drawn in at the face opening and is immediately drawn through the front grille and under the work surface. The air is then blown through

the rear air plenum to the top of the cabinet where it is divided into two chambers. Thirty percent of the air is exhausted out of the cabinet through a high efficiency particulate air (HEPA) filter into the laboratory room. The remaining seventy percent of the air is directed through another HEPA filter down onto the work surface in a laminar flow directional air pattern. The typical HEPA filter removes 99.97% of all particles that are 0.3 micron or larger in size, which means that all microbial agents will be trapped in the filter. The air returned to the laboratory and delivered to the work surface is virtually sterile.

Infectious waste materials should be chemically disinfected or, preferably, decontaminated in a steam autoclave. Infectious waste materials to be removed from a BSC should be placed in a pan or tray that can be covered during transport to the autoclave, or placed in a biohazard autoclave bag. By placing an inch or two of water in the bag before sealing it for transport, steam will be generated within the bag during the autoclave cycle. Laboratory personnel should be aware of the potential hazards associated with the work and be proficient in the specified practices and procedures.

5.5 Biosafety level - 3 (BSL-3)

There are some specific secondary barriers needed at BSL-3, that tend to set these laboratories apart from BSL-2. These laboratories are characterized by having a double-door entry. Because the agents manipulated at BSL-3 are transmissible by the aerosol route, particular attention is given to air movement in these laboratories. Air moves from areas of lesser contamination to areas of higher contamination, such as from the corridor into the laboratory. Air movement is also single pass; exhaust air is not recirculated to other rooms. Exhaust air does not have to be HEPA filtered, unless local conditions are such that reentrainment into building air supply systems is unavoidable.

Standard microbiological practices are the same as for BSL-1 and BSL-2 laboratories. Class II type A biological safety cabinets are suitable in BSL-3 laboratories.

5.6 HIV and biosafety

The risk of acquiring HIV following percutaneous exposure (needle stick/prick with inoculation) from a source (blood or patient) positive for HIV is extremely low 0.25 to 0.3%. This is because the concentration of HIV in peripheral blood is extremely low (10–100 infectious doses/mL). Whereas, the risk of acquiring HBV following similar expsoure ranges from 9–30% because the concentration of HBV in blood is very high (> 10,000,000 infectious doses/mL). The chance of acquring hepatitis C virus (HCV) is approximately 3–10%. Risk of transmission of HIV following muco–cutaneous exposure is 0.05 %.

HIV is one of the infections associated with exposure to contaminated blood/ body fluids, others being viral hepatitis (HBV, HCV, HDV, HGV), HTLV–1 and HTLV–II, CMV, viral haemorrhagic fevers, E.B. viruses and many others. All infections acquired through contaminated blood can be effectively prevented by the medical professionals by diligent practice of "standard work precautions (S.W.P.)" and or "Biosafety precautions."

Most important and common practice is to presume that all specimens, all patients / clients are infected and potentially infectious until proved otherwise. This is so because the correct HIV status of an individual can be known only by laboratory testing, to do which is not feasible and not cost effective. It is better to practice "S.W.P." at all times while providing medical care services in all kinds of health practices for all patients and all specimens.

5.7 Magnitude of risk to HCW

Prospective studies demonstrate that risk of HIV per episode of percutaneous exposure to infected blood is approximately 0.3% (i.e 1 per 300 exposures for HIV). Whereas, risk of acquiring HBV is 9–30% and HCV is 3–10%. Risk after mucocutaneous exposure is 0.05%.

- Risk depends on prevalence of infected individuals in the population
- Frequency of exposure to contaminated sharps etc.
- Relative infectivity of the virus
- Concentration of the virus in the blood
- Deep needle stick injury with inoculation of blood.
- Source patient who has AIDS or died within 60 days after the exposure occured.

5.8 The following materials have been found to be infectious and thus require precautions in handling

Blood is the single most important source of HIV, HBV and other blood borne infections for HCWs. Although semen and vaginal secretions have been implicated in sexual transmission of HIV and HBV they have not been implicated in occupational transmisison to HCW, since chances of exposure to such infectious materials in health care setting are limited. HIV has been isolated from CSF synovial and amniotic fluid and HBsAg has been detected in synovial fluid, amniotic fluid and peritoneal fluid. Documentation in implicating these fluids in transmission of HIV and HBV in health care setting is uncertain.

HIV and HBsAg have been documented in specimens like faeces, nasal secretions, sputum, tears, urine, vomitus. However epidemiologic studies in health care setting have not implicated these materials in transmission of infection.

Breast milk

Human breast milk has been implicated in perinatal transmission of HIV, and HBsAg has been found in the milk of mothers infected with HBV. However, occupational exposure to human breast milk has not been documented for HIV or HBV. Moreover the HCW will not have the same type of intensive exposure to breast milk as the one nursing the neonate. Nevertheless, gloves should be worn by HCWs in some settings e.g. breast milk banking. When the identity of the specimen in case of exposure is not known, it is advisable to treat all specimens as infectious.

Biosafety practices in a health care setting include

- Practice of standard work precautions at all times while providing services.
- Effective disinfection and sterilization.
- Safe disposal of hospital waste.
- Immunization for HBV.

5.9 Universal work precautions (UWP)

UWP are certain protective measures to be practiced by all the H.C.W. at all times while providing professional services, mainly directed at blood & body fluids & tissues to minimize the risk of HIV/HBV transmission.

Protective measures properly applied will

- Prevent occurrence of accidental exposure & transmission of infection
- Control surface contamination
- Ensure safe disposal of contaminated waste

Components of U.W.P.

General blood and body fluid precautions

- Hand washing
- Careful handling of sharps
- Safe techniques
- Sterilization
- Disinfection
- Disposal of disposables/reusables : as appropriate
- Adherence to correct hospital sterilization & disinfection protocols
- Use of personal barrier precautions (gloves, masks, gowns/aprons, protective eyewear, foot cover)
- HBV immunization of HCW at risk i.e. those in contact with blood/body fluids.

HIV and the Environment

- HIV was recoverable by tissue culture techniques up to 3 days after drying in laboratory studies (concentration of virus is 100,000 times greater than found in blood of HIV infected person).
- CDC, U.S.A. studies have shown that drying causes a 90–99% reduction in HIV concentration within several hours.
- In tissue culture fluid, cell free HIV could be recovered upto 15 days at room temperature, upto 11 days at 37°C and upto 1 day if HIV was cell associated.
- No one so far has been HIV infected as a result of contact with an environmental surface.
- HIV cannot reproduce outside the living host except under laboratory conditions and cannot spread or maintain infectiousness outside its host.

5.10 Occupational exposure and post exposure management

Contact with known HIV/HBV infected material resulting from :

- Percutaneous inoculaton (Needle stick, cut with a sharp etc)
- Contamination of an open wound
- Contamination of breached skin (chapped, abraded, dermatitis)
- Contamination of a mucous membrane including conjunctiva

Post exposure management

The risk of infection varies with the following factors

- Type of exposure e.g. exposure of intact skin, nonintact skin, mucous membrane or needle stick injury.
- The amount of blood involved in the exposure; hollow bore needles, canula etc. carry more blood than IM needles.
- The amount of virus in patients blood at the time of exposure (maximum during viraemia).
- Prevalence of infection in the population
- Number of exposures in case of needlestick injuries from individuals with unknown serostatus.
- Timely availability of post exposure prophylaxis (PEP).

Allow to bleed Wash with water Apply antiseptic.	Minor bleed with percutaneous inoculation, open skin wound, breached skin, wound, breached skin, exposed mucous membranes.
Report	Employee indentification date, time & place of accident cirumstances around accident and action taken.
Initial consultation	Easy access to medical advice & counselling.
Laboratory testing	After consent & counselling within 2 weeks, 6 weeks, 12 weeks, and 24 weeks.
Clinical follow up	For fever, Pharyngitis, Rash, Malaise, Lymphadenopathy, Myalgia, Arthralgia within 6 months. If the HCW tests negative 1 year after the accident means that the HCW is not infected.

Post exposure prophylaxis should be given as per the recommendations of NACO (Chapter –6).

5.11 Spills and accidents (management)

- Wear gloves throughout.
- Cover spill with absorbent material and pour disinfectant around the spill and over the absorbent material. Leave for 30 min. Clean with absorbent material and place in contaminated waste container.
- Wipe the surface again with disinfectant.
- Sweep broken glass etc with a brush into the waste container.
- Needle stick, puncture wounds, cuts, open skin contaminated by spills or splashes should be washed with soap and water. Encourage bleeding from the wound.
- Report all spills/accidents to the supervisor.
- Keep a written record of all such accidents.
- Appropriate medical evaluation, surveillance, treatement and counselling should be provided.

5.12 Serological laboratories : biosafety precautions

- Clearly identified adequate area should be provided either separately or within the laboratory.
- Bio-safety cabinet is not required for serology of HIV.
- Protective clothing, shield and glasses to be used to protect eyes and face.

- Bench tops should be impervious and resistant to disinfectants.
- Walls, ceiling and floor should be smooth, easy to clean and impermeable and resistant to disinfectants.
- Wash basins in each Laboratory near exit.
- Doors should be self closing.
- Autoclave, waste boxes and incinerator should be available on premises.
- Separate space for eating, drinking, dressing and smoking etc.

5.13 Transport of specimen by public conveyance

- Transport in leak proof, properly labelled containers in upright position.
- Wrap primary container in enough absorbent.
- Primary container placed in secondary container which should be strong enough to withstand physical damage in transit.
- Make arrangements for dispatch and collection in advance.
- Specimen data forms, letters and other relevant information are taped on the outside of secondary container.
- National/international shipping regulations to be observed.

5.14 Sterilization and disinfection

5.14.1 Sterilization method

Sterilization is a process which destroys all microorganisms (bacterial, viral, parasitic and fungal) including the resistant spore forms. Dry and moist heat, certain gases and chemicals are used for sterilization.

- Autoclaving at 121°C for 20 mins. at 15 lbs pressure
- Dry heat 170°C for 1 hour (holding time)
- Boiling for 20–30 minutes

5.14.2 Chemical disinfectants

Disinfection is a process which destroys the infectious microorganisms. High level disinfection destroys all pathogenic organisms and most of other microorganisms but spores may survive. Intermediate and low level of disinfection destroys the pathogenic organisms. Decontamination is the same as low level of disinfection, this process gets rid of visible, contamination of surfaces, equipments etc.

- Sodium hypochlorite : 1gm/L
- Calcium hypochlorite : 1.4 gm/L
- Chloramine : 20 gm/L

  Available chlorine 0.1%, Chloramine is most stable of the above three disinfectants
- Ethanol 70%.
- Povidone iodine (PVI)
- Formaline : 3-4%
- Glutaraldehyde : 2% for 30 minutes

5.15 Safe disposal of wastes

Hospital wastes are potential hazards. Infectious waste can transmit numerous diseases in the community and put those who handle waste and live in proximity, at risk. Besides, the increasing use of disposables in health care is also posing an additional burden on the waste management facility. It is extremely important that the recycling of these items is prevented. Only a small percentage (< 10%) of the waste generated in health care settings are infectious while another 5% is non-infectious but hazardous. The most practical approach to the management of biomedical waste is to identify and segregate infectious waste (with a potential for causing infection during handling and disposal), for which some special precautions appear prudent. This will drastically reduce the cost of the disposal methods in health care settings.

Setting up of biomedical waste facility

Every hospital, nursing home, veterinary institution, animal-house, blood banks, research institutes generating biomedical wastes should install an appropriate biomedical waste facility in the premises or should set up a common facility in accordance with the directions given by the appropriate authority. Biomedical waste should not be generated without authorisation. Every hospital should have a waste management programme. Waste survey is an important part of the waste management programme and helps in determining both the type and quantity of waste being generated in the hospital including the laboratory and determine the feasible methods of disposal.

Containing waste at generation point

At the generation point i.e. the laboratory, waste is managed in the following way :

i) Collection

ii) Segregation and weighing

iii) Storage

Waste segregation is the key to any waste management scheme. It consists of placing different types of waste in different containers or colour-coded-bags at the site of generation. This helps in reducing the bulk of infectious waste and contains spread of infection to general waste. This practice reduces the total treatment cost, the impact of waste in the community and the risk of infecting workers. Proper segregation should identify waste according to source and type of disposal/ disinfection.

Waste should be segregated into different categories at the site of generation i.e in the laboratory and weighed separately at the time the waste is being disposed.

5.15.1 Segregate waste at source into

- Solid noninfectious household type waste e.g. paper, fruit peels etc. and dispose off in the routine dustbin/black bags and final disposal into the MCD bin,
- Infected sharp waste disposable e.g. disposable syringes -needles and other sharps ⟶ place in a puncture resistant container containing disinfectant (0.1%-0.5% bleach solution). The container should be placed near the activity place.
- Infected non sharp disposable waste e.g. catheters , gloves etc. ⟶ place in a container containing 0.1.-0.5% sodium hypochlorite solutions.

- Infected reusable instruments e.g. endoscopes, speculum etc. ⟶ place in a container containing 2% glutaraldehyde for 30 minutes ⟶ wash and autoclave or place in 2% glutaraldehyde for 6–8 hours as per the specifications of the instrument.
- Swabs– should be chemically disinfected followed by incineration.
- Disposable items include single use products (syringes, gloves, sharps etc.)– As these items are often recycled and have the risk of being reused illegally, these should be disinfected by dipping in freshly prepared 1% Sodium hypochlorite for 30 minutes to 1 hour. Containers which can be used for this purpose are a set of twin containers, one inside the other with the inner one being perforated and easily extractable. This minimizes contact when the contents are being removed.

Disposable items like gloves, syringes etc. should be shredded, cut or mutilated before disposal followed by deep burial or properly accounted before disposal. Extreme care should be taken while handling the needles.

Liquid wastes generated by the laboratory are either pathological or chemical in nature and are disposed of as follows:

- Non–infectious chemical waste should first be neutralised with reagents and then flushed into conventional sewer system.
- The liquid infectious waste should be treated with a chemical disinfectant for decontamination then neutralized and flushed into the sewer.

Collection bags. Solid wastes are collected in leak–resistant single heavy duty bags or double bags. Bags having different colour codes (Table) with red labels mentioning date and details of waste are recommended. The bags are tied tightly after they are three–fourths full.

5.15.2 Packing , storage and transport

- All segregated and disinfected waste should be packed in proper containers and colour–coded bags (Table) with red labels mentioning details of biomedical waste and biohazard signs. All containers used for storage of such waste should be provided with a properly covered lid.
- Such containers should be inaccessible to scavengers and protected against insects, birds, animals and rain.
- There should not be any spillage during handling and transit of such waste.
- The sharp wastes, after pre–treatment should be broken before packing in the container.
- The waste should be transported in vehicles authorised for this purpose only.
- No such waste should be stored in the place where it is generated for a period of more than two days.

5.15.3 Treatment and disposal

Disposal methods: Disposal may be done by :

- Municipal corporation.
- Sanitary landfill.
- If incinerator is not available, deep burial in controlled landfill sites is recommended. Decontamination should be carried out before burial.

Incineration (Temp.750°C) Incinerator- burns /reduces the infectious waste to ashes and therefore favoured by hospitals. It may be of two types -common or individual. There are some disadvantages like pollution /incomplete melting of needles.

Container and colour coding for disposal of bio-medical wastes

Waste category	Waste class	Type of container	Colour coding	Treatment / Disposal option
No. 1	Human anatomical waste, blood & body fluids	Single-use containers/ Plastic holding bags	Red	Incineration / Deep Burial
No. 2	Animal & slaughter house waste	Single-use containers/ Plastic holding bags/ sacs	Orange	Disinfection & Deep Burial
No. 3	Microbiology & bio-technology waste	Single-use containers/ Plastic holding bags	Yellow	Autoclaving / Microwaving & Incineration
No. 4	Waste sharps	Re-usable/ single-use sturdy containers of plastic, glass or metal	Blue	Shredding & Deep Burial
No. 5	Discarded medicines	Re-usable/ sturdy cardboard/ glass/ plastic holding bags	Blue	Shredding & Deep Burial
No. 6	Soiled wastes (linen)	Plastic bags/ sacs	Yellow/ Black	Disinfection and machine cleaning
No. 7	Disposables (other than sharps)	Re-usable/ sturdy containers/ plastic holding bags	Yellow/ Black Burial	Disinfection-Chemical / Autoclaving, Shredding,
No. 8	Liquid wastes	NA	NA	
No. 9	Chemical wastes	Sturdy containers/ plastic holding bags	Yellow/ Black	

5.15.4 Implementation of biosafety practices

Although guidelines regarding universal precautions and other biosafety practices are available since long.

Strict implementation is eventually not in practice in healthcare settings in India. With increase in the prevalence of HIV infection, there is a definite need that the HCW's take biosafety practices seriously. For effective compliance, the hospital managers should ensure adequate supply of personal protective equipments, availability of materials for handwashing, disinfectants and set up an effective waste disposal programme for disposal of biomedical wastes.

5.16 References :

1. Medical Management of HIV Infection 1998 edition, Editor John G. Bartlett John Hopkins University Division of Infectious Diseases, USA.

2. Global Programme of AIDS-Report of a WHO Consultation on the prevention of Human Immunodeficiency Virus and Hepatitis B virus transmission in health care setting Geneva, 11-12 April, 1991

3. Ministry of Environment and Forests notification, New Delhi the 16 th October, 1997.

4. Shrishti : Implementing Hospital Waste Management : A guide for health care facilities. September, 1996.

5. WHO Laboratory Bio-safety Manual 2nd edition, 1993 World Health Organisation, AITBS Publishers and Distributors.

6. WHO Managing Medical Waste in Developing Countries, 1994 World Health Organisation, Geneva.

7. AIDS in India. News letter of the National AIDS Control Organisation, September, 1993.

8. Perspectives in Disease Prevention and Health Promotion update Universal Precautions for prevention of transmission of Human Immunodeficiency Virus, Hepatitis B Virus and other Blood-borne Pathogens in Health-care Setting. MMWR 37(24), 377-388.

9. Centers for Disease Control. Recommendations for prevention of HIV transmission in health-care settings. MMWR 1987 : 36 (suppl no. 2S).

Chapter –6

POST EXPOSURE PROPHYLAXIS

6.1 Introduction

Health Care Workers (HCW) working at different levels (Safai Karamchari to Director Professor) in different disciplines of medicine come in direct or indirect contact with patient, patient's blood and other potentially infectious materials (OPIM), body fluids, and contaminated fomites while providing services and hence are at risk of acquiring HIV. The risk of occupational exposure though, minimal at the moment may increase with increase in prevalence of HIV/AIDS and if the standard work precautions (SWP) are not practised.

6.2 Quantum of risk

The quantum of risk depends upon the discipline of medicine the HCW is working in, the type of patient population that attends the hospital and the location of the health care setting as well as the knowledge and aptitude of the HCW towards practice of standard work precautions. The risk factors which determine the frequency of occupational exposure and risk of acquiring HIV infection are given below :

(i) Frequency of occupational exposure

- Contact with blood and body fluids
- Accidental needlesticks/sharp instrument injuries

(ii) Occupational area in the hospital

- Haemodialysis
- Laboratory services departments (Pathology, Microbiology, Biochemistry, Clinical Laboratories etc).
- Blood bank
- Dentistry/oral surgery

(iii) Geographic location of health care setting

- Urban
- Rural

(iv) Patient population attending the facility

- Haemodialysis
- Intravenous drug users
- Homosexual men
- Prison inmates
- Developmentally disabled
- Immigrants from highly endemic areas

(v) Source infectivity

- High : AIDS patient with low CD4 cell count.
- Low : Asymptomatic individual or on antiretroviral treatment with high CD4 count

Most of occupational HIV infections are acquired through percutaneous injuries. The overall risk is estimated to be one in 330 i.e. 0.3% approximately. The overall risk after mucous membrane and skin exposure is around 0.05 to 0.1%

6.3 Occupational exposure

It is defined as an event/occurrence/episode which may place a HCW at risk of acquiring HIV infection while providing services. Exposure may be a percutaneous injury, contact of intact skin/ intact mucous membrane and compromised skin (chapped, abraded, dermatitis etc.) with blood and other potentially infectious body fluids to which SWP apply.

Most exposures do not result in HIV infection. Even so wherever post exposure prophylaxis (PEP) is indicated it should be taken to prevent HIV infection. It has been shown in some of the experimental studies in animals that PEP either prevented or ameliorated the HIV infection. Also the fact that even single dose antiretrovial therapy in HIV infected mother reduced mother to child transmission (MTCT) rate of HIV supports the rationale of use of PEP. Even single dose of nevirapine to the mother during labour and newborn in 48 hours has been found to reduce MTCT of HIV-1. How HIV-1 infection is prevented by PEP in HCW and in the newborn by antiretroviral drug therapy is not exactly known. May be it is by reduction in the viral load in the blood. PEP may not offer absolute protection. However, it is better to take PEP following exposure wherever indicated. Decision to take PEP depends upon the HCW and the level of exposure.

6.4 National guidelines

All accidental exposures to blood and body fluids do not result in HIV infections. It is again empahsized that the risk is as low as 0.3% for percutaneous exposures, 0.1% for mucous membrane and skin exposures (approximately). Whenever there is exposure to blood or other potentially infectious materials following steps should be taken for management.

- Do not panic
- Allow the area/wound to bleed under running water, wash area with water.
- Apply disinfectant
- The accident should be reported to the appropriate authority (Member Secretary, Hospital Infection Control Committee etc.) who notes the date, time place and circumstances around the accident, i.e. HCW identification, patient identification (relevant facts) and the action taken.
- HCW is counselled and medical management advice is given.
- Blood of HCW is collected after counselling and consent within 2 weeks and after 6 weeks, 12 weeks and 6 months following the accident for HIV testing, particularly if the first sample is HIV negative.
- Level of exposure should be assessed immediately to decide whether PEP is required or not. This is done by estimating the exposure code in HCW, HIV status code of the blood or OPIM to which the exposure has occurred and the two are collated to decide whether PEP is required or not. The details of this are shown in Table-6.1,6.2 and 6.3.

- Clinical follow up of the HCW should be done. Occurrence of fever, pharyngitis, rash, malaise, lymphadenopathy, myalgia and arthralgia etc. within 6 months following the exposure is noted.
- If the HCW tests HIV negative 6 months after the exposure that means HCW is not infected.

Table 6.1 Determination of the Exposure Code (EC)

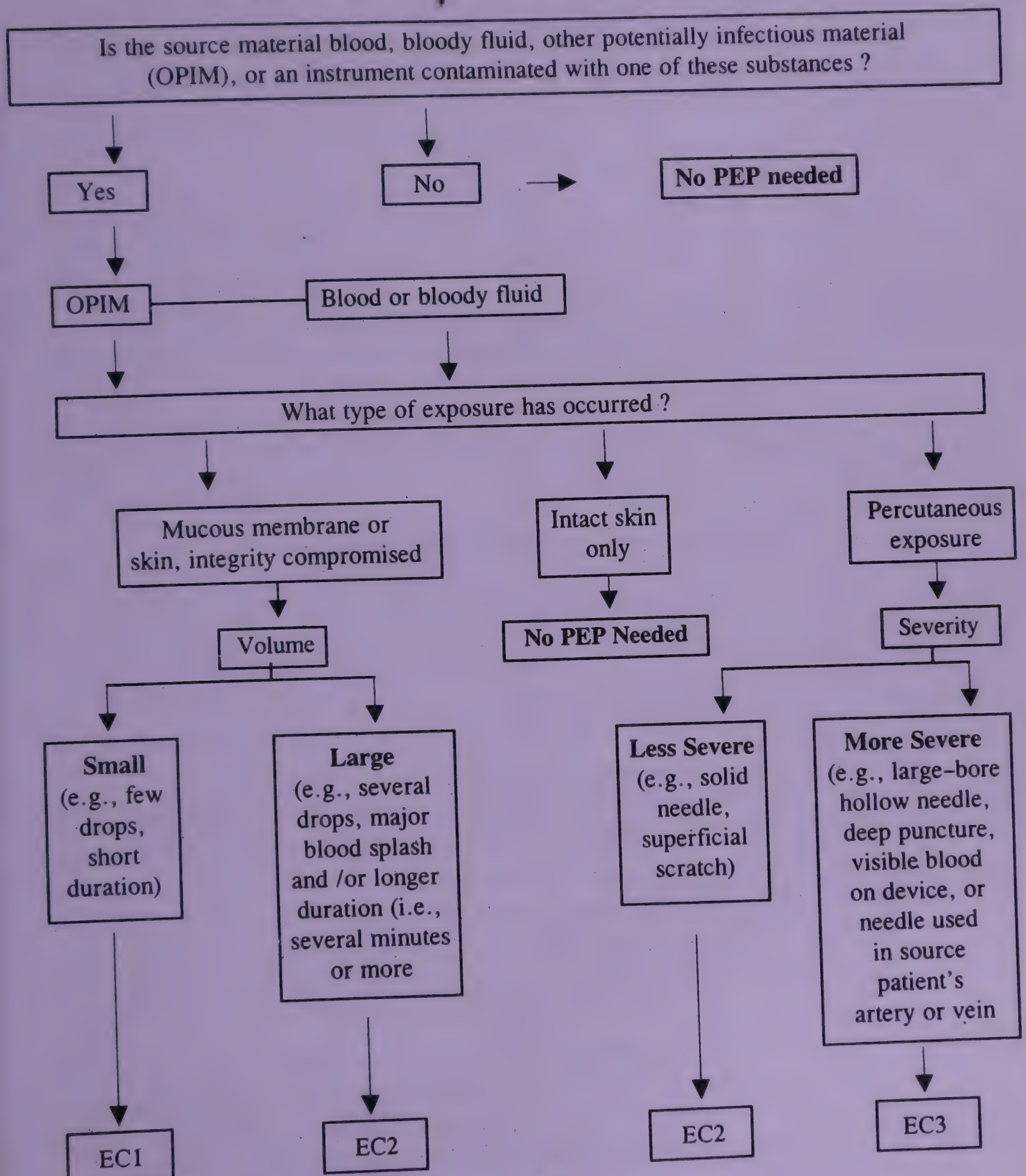


Fig. 6.2 Determination of the HIV Status Code (HIV SC)

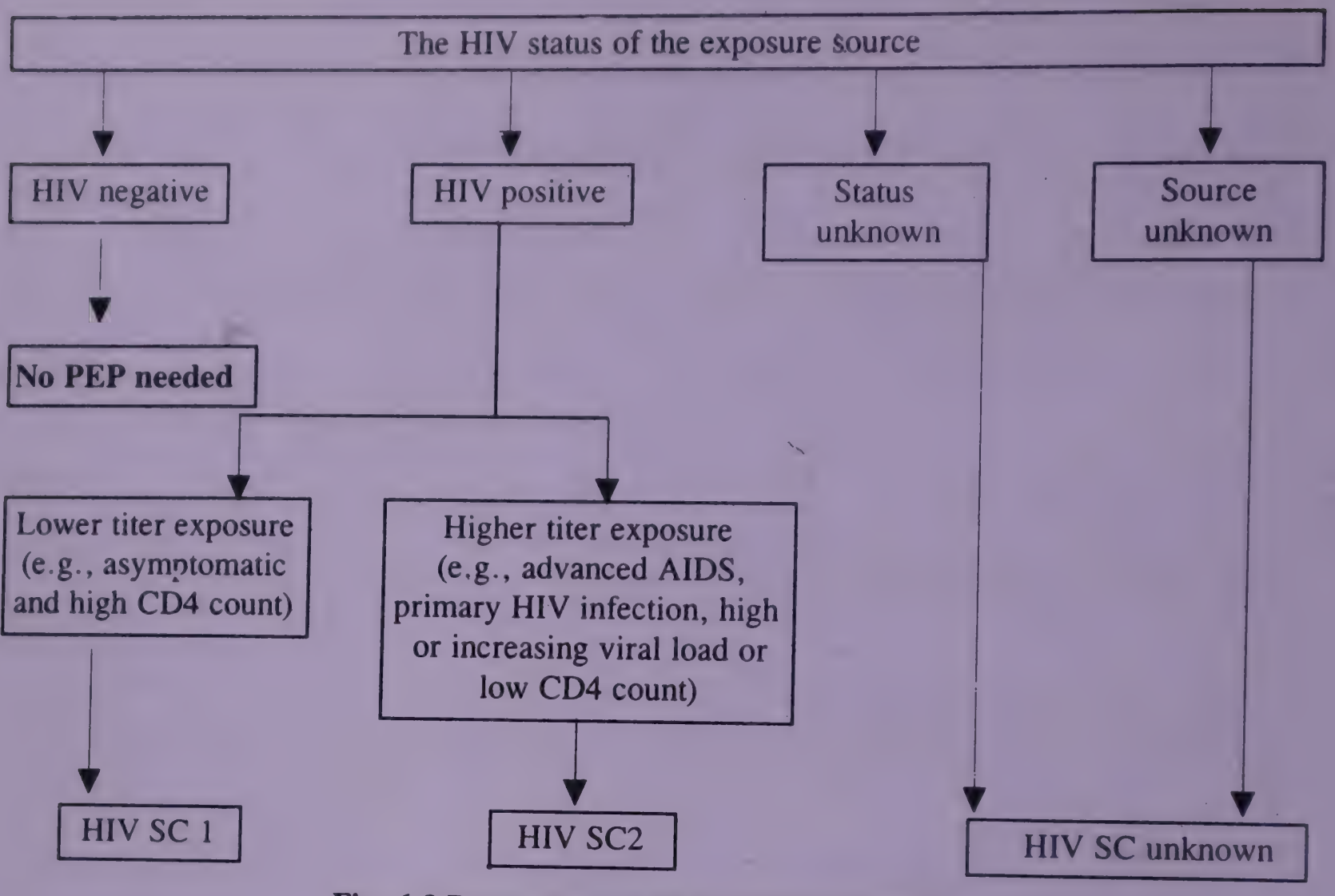


Fig. 6.3 Determine the PEP recommendation

EC	HIV	SC PEP recommendation
1	1	PEP may not be warranted
1	2	Consider basic regimen. Exposure type poses a negligible risk for HIV transmission.
2	1	Recommend basic regimen Most HIV exposures are in this category ; no increased risk for HIV transmission has been observed but use of PEP is appropriate.
2	2	Recommend expanded regimen. Exposure type represents an increased HIV transmission risk.
3	1 or 2	Recommend expanded regimen. Exposure type represents an increased HIV transmission risk.
	UNKNOWN	If the source, (in the case of an unknown source), the setting where the exposure occurred suggests a possible risk for HIV exposure and the EC is 2 or 3, consider PEP basic regimen.

6.5 Anti-retroviral drugs and regime for PEP

Zidovudine has been widely used for PEP. However, monotherapy is not recommended by National AIDS Control Organisation, Government of India on account of development of drug resistance and failure of PEP. Combination therapy, either basic regime or expanded regime is recommended by NACO. Basic regime comprises of two nucleoside analogue reverse transcriptase inhibitors (NRTIs) i.e. Zidovudine, and lamivudine. The expanded regime to be given in cases of severe exposure comprises of two RTIs as above with addition of one protease inhibitor (PI) which may be Indinavir or Ritonavir etc.

Dose schedule used for PEP

Zidovudine	200 mg. three times a day
	or
	300 mg. twice a day
Lamivudine	150 mg. three times a day
Indinavir	800 mg. three times a day
Ritonavir	600 mg. two times a day

The antiretrovirals for PEP are to be given for four weeks. Government of India has already made the money available with various State AIDS Societies to meet with the expense of PEP for HCWs.

The report of exposure and PEP has to be sent to Joint Director (Technical), National AIDS Control Organisation, Ministry of Health & Family Welfare, Nirman Bhavan, New Delhi - 110011.

6.6 Conclusions

The risk of transmission of HIV following accidental exposure is extremely low. Even so, prompt PEP should be given if indicated to prevent transmission of HIV to HCWs. The practise of standard biosafety and work precautions is of paramount importance to prevent the occurrence of accidental exposure. PEP can be taken by pregnant health care workers also. Confidentiality of HCW found to be infected must be maintained. All exposures must be reported to the identified Officer (Local) and to National AIDS Control Organisation, government of India as above.

6.7 References :

1 Guay LA, Musoka P, Fleming T, et al. Intrapartum and neonatal single dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda : HIV/NET 012 randomised trial. Lancet 1999; 354 : 795-802.

2. Easterbrook P, Ippolito G. Prophylaxis after occupational exposure to HIV. Recent Guidelines should promote good practice and data collection. Br. Med. J 1997; 315 : 557-558.

3 Centres for Disease Control. Case control study of HIV seroconversion in Health care workers after percutaneous exposure to HIV infected blood - France, United Kingdom, and United States, January 1988 - August 1994. MMWR 1995; 44 : 929-33.

4. Saag MS. Candidate antiretroviral agents for use in post exposure prophylaxis. Am J Med. 1997; 102 (suppl) : 25-31.

Chapter –7

INFRASTRUCTURAL FACILITIES FOR LABORATORY DIAGNOSIS OF OPPORTUNISTIC INFECTIONS IN HIV/AIDS

7.1 Introduction

Early accurate diagnosis and management of different opportunistic infections is extremely important for the proper management of HIV infected people. Laboratory plays an important role in this. These infections can be either Bacterial, Parasitic, Viral or Fungal in nature or a combination of any of the above.

The diagnosis of common infections at PHC/CHC level is based on simple staining techniques like Gram staining, Z-N staining and direct microscopic examination. The staff, space and equipments/ reagents required for this mostly exist. However, minimal inputs in the form of additional reagents may be required as given in the tables.

The district level laboratory can in addition to the functions performed at PHC also undertake culture and identification of common microorganisms (bacterial) and serodiagnostic techniques. These laboratories can be strengthened with minimal inputs like filling up of the vacant posts whereever required, provision of equipments and reagents as shown in the tables.

Most of the major hospitals and medical colleges have adequate infrastructure (space, staff, equipements, reagents) to undertake the diagnosis of bacterial, parasitic and fungal infections, however, some inputs may be required for the laboratory diagnosis of viral infections e.g. fluroescent microscopes, biosafety cabinets, inverted microscopes etc.

The National Reference Centres should be strengthened to provide referral diagnostic services in difficult cases and conduct training courses for staff from states and medical colleges.

The ideal space, staff, equipments and reagents etc. which are required for diagnosis of common opportunistic infections is given in the following tables.

The medical laboratory specialists and the supporting laboratory staff should be trained in different microbiological techniques at the appropriate reference centres.

The staff already exists at most of the laboratories however wherever posts are lying vacant they should be filled up.

The space requirements mentioned above may be already existing in most of the laboratories however in case it is not there one should try to make it available as per above standards.

The staff, equipments and reagents mostly exist in Medical Colleges. Some or all of these colleges can be identified to provide diagnostic services for OIs with some additional imputs. Reference centres already existing in different zones of the country can be strengthened with few inputs to provide these services.

7.2 Types of laboratory procedures to be carried out at different levels of laboratories

Level of Lab.	Category of opportunistic infections				
	Bacterial	**Mycobacterial**	**Parasitic**	**Fungal**	**Viral**
PHC/CHC	- Gram staining	- AFB staining only	- Wet films (Direct Microscopy) - Modified acid fast staining - Giemsa staining	- Gram staining - KOH preparation	- ollection of blood/ serum, other samples for onward ransmission to higher level lab.
District Laboratory	- Gram staining - Culture and identification - Serology	- AFB staining only	- Wet films (Direct as well as concentrated) - Giemsa staining - Modified acid fast staining - Trichrome staining	- As above	- Giemsa staining - Rapid kit based serology
State Level Laboratory	- Gram stain - Culture and identification - Serotyping - Serology - Biotyping - Antibiogram	- AFB staining - Fluorescent staining - AFB culture - Antibiotic sensitivity	- As above + - Toluidine blue staining + - Serology + - Fluorescent staining	- As above + Culture	- As above + - Viral culture + - Serology
National / Reference Laboratory	- As above + - Any special typing procedure - PCR (Polymerase chain reaction confirmatory test wherever required)	- AFB culture and sensitivity	As above +	- All above + - Culture + - Biochemicals + - Drug sensitivity + - Serology	- All above + - Serotyping + - Genetic typing + - PCR

7.3 Staff requirements at different levels of laboratories

	PHC	District	State	National/Reference
(I)	One technician	One qualified Microbiologist or Pathologist	One to two qualified Microbiologists	One Senior Consultant Microbiologist/division
(II)	One laboratory assistant/attendant	One or two experienced technicians preferably having certificate or diploma in MLT	One/two Junior level doctors (MBBS) – for performing invasive procedures for collection of specimens (BAL, FNAC, Biopsy etc.)	Two/three Qualified Junior Microbiologists /division
(III)		One laboratory assistant	One technician with MLT qualification, one /lab.	One/two Junior level doctors (MBBS)
(IV)		One laboratory attendant	One laboratory assistant/lab.	Laboratory assistant, two/ three/division
(v)		One safaiwala	One laboratory attendant/lab.	Laboratory attendant, two/three/division
(VI)		One clerk cum store keeper	One safaiwala/lab.	Safaiwala, two/three/ division
(VII)			One clerk cum store keeper	Clerk cum store keeper,one/division

7.4 Space requirements at different levels of laboratories

	PHC	District	State	National/Reference
(I)	One laboratory cum office room (size = approx. 5 mts. x3 mts)	Minimum one main laboratory room of size (8 mts. x 5 mts.)	Separate room each of size (8 mts. x 5 mts.) for – Bacteriology – Tuberculosis – Parasitology – Mycology – Virology – Training room	At least 2 rooms each of size (8 mts. x 5 mts.) for each of the following Divisions – Virology – Bacteriology – Mycology – Parasitology – Tuberculosis
(II)	One store room (5 mts. x 3 mts.)	One media preparation room (5 mts. x 4 mts.)	Sterilization room (6 mts. x 4 mts.)	Sterilization / decontamination room (6 x 4 mts.) for each Division
(III)		One sterilization room (5 mts. x 4 mts.)	Media preparation room (6 mts. x 4 mts.)	Medial preparation room (6mts.x4 mts.) for each Division
(IV)		One office–cum–record room (5 mts. x 3 mts.)	Washing room (6 mts. x 4 mts.)	One washing room (4 mts. x 4 mts) for each Division
(v)		One store room (4 mts. x 3 mts.)	One room (4x4 mts.) for fluorescent microscopy	One PCR room (4 mts. x 4 mts.) for each Division
(VI)			One room (5x4 mts.) for carrying out cell cultures	One store room (5 mts. x 4 mts.) for each Division

	PHC	District	State	National/Reference
(VII)			One room (4x4 mts.) for doing PCR	One office room for each Division (4x3 mts.)
			Record cum reporting room (5x4 mts.)	Record cum reporting room (5x4 mts.)
			Training room (8 mts. x 5 mts.)	Staff room for the Division
			One store room (4x3 mts.)	A common training room (10 mts. x 8 mts.)
			Staff room (8x6 mts.)	If possible a room with biosafety level – 3 facility to handle highly infectious organisms details given in Chapter - 5)

7.5 Reagents/Chemicals/ Stains required at different levels of laboratories

PHC	District	State	National/Reference
– 4% KOH Reagents for – Gram staining – Z–N staining – Giemsa staining – Sterilized disposable containers for sputum stool, blood and other samples. – Sterilised syringes and needles	– 4 % KOH Reagents for different types of staining – Formol ether concentration /other concentration techniques – Gram staining – Z–N staining – Giemsa staining – Modified acid fast staining – Toluidine blue staining – Trichrome staining – Culture media for routine cultures – Rapid serology test kits for antigen detection – Sterile syringes, needles – Sterile, disposable sample containers	As in District laboratory + – Reagents for complete serology (Bacterial/viral) – Antisera for serotyping of bacteria, viruses – Culture media to cover all opportunistic bacteria, including Mycobacteria – Reagents for cell culture for virus propagation – Reagents for fungal culture – Reagents for drug sensitivity tests – Reagents for fluorescent and immunofluorescent staining – Trichrome staining	As in State level + – Reagents for carrying out PCR for different pathogens + – Reagents and media for Bactec 460 TB system + – Reagents for fungal serology

7.6 Major equipments required at different levels of laboratories

	PHC	District	State	National/Reference
(I)	Good quality compound microscope (1–2)	Binocular compound microscope with in built light system (2–3) ·	Same as at district level + – Fluorescent microscope – Inverted microscope – ELISA reader – Deep freezer (–70° C) – BSL–2 biosafety Cabinets (details given in Chapter-5) - Liquid nitrogen containers	Same as at state level + – Equipments for doing PCR test PCR test e.g. Thermal cycler Electrophoresis apparatus Photographic equipment – BSL - 3 biosafety cabinets (details given in Chapter - 5)
(II)	Table top centrifuge with speed upto 5000 rpm	Centrifuge with speedometer, timer and sealed buckets		
(III)	Vertical autoclave	Vertical autoclaves		
(IV)	Refrigerator, domestic	Hot air oven		
(V)	Balance	Incubator		
(VI)	Water bath	Water bath		
(VII)	Incubator	Refrigerator		
(VIII)	Burner/spirit lamps	Inoculating chamber		
(IX)		Balance		
(X)		pH meter		
(XI)		Distillation plant		
(XII)		Micropipettes		
(XIII)		Deep freezer (–20°C)		

7.7 List of common opportunistic infections associated with HIV/AIDS.

A. Bacterial infections

- Mycobacterium tuberculosis
- M. avium complex
- Salmonella spp.
- Bacteria producing pneumonias
- Campylobacter spp.
- Nocardia spp.
- Listeria monocytogenes
- Shigella spp.
- Legionella pneumophila

B. Parasitic infections

- Giardia lamblia
- Cryptosporidium
- Isospora belli
- Toxoplasma gondii
- Strongyloides stercoralis
- Entamoeba histolytica
- Microsporidium

C. Viral infections

- Cytomegalovirus
- Varicella zoster virus (VZV)
- Herpes simplex virus (HSV)
- J.C. virus (Progressive multifocal lencoencephalopathy)

D. Fungal infections

- Candida spp.
- Cryptococcus neoformans
- Histoplasma capsulatum
- Coccidioides immitis
- Pneumocystis carinii
- Penicillium marneffei
- Trichophyton spp.
- Epidermophyton
- Sporothrix schenkii

Chapter –8

COLLECTION AND TRANSPORT OF SPECIMENS

HIV Infected immunocompromised individuals can suffer from almost any infection endemic in the community. So, the type of specimen to be collected will depend upon the type of infection and the system involved. The collection and transport of specimens like sputum, faeces, body fluids etc. are described in the different sections. However, as most of the laboratory procedures for HIV/AIDS associated infections are performed on patient's blood, serum or plasma the collection, transport and storage of these samples is described below.

8.1 Performing venepuncture

- Gloves should be worn and sterilised/disposable syringes and needles should be used.
- For avoiding soiling, a piece of linen with a layer of dressing pad (a sheet of absorbent cotton between two layers of gauze piece) or simply a big piece of absorbent cotton may be placed below the forearm before commencing vene-puncture.
- After collecting 5 ml. of blood aseptically, it should be carefully transferred from the syringe without squirting in to a sterile plastic leak proof specimen container, preferably screw capped. The container should be labelled before commencement of venepuncture. If the vial has anticoagulants, then a second person wearing gloves would have to help in shaking the vial for mixing the blood well with the anticoagulants. The cap should be tightly screwed after the blood has been transferred to the vial.
- After blood is collected, and the needle is withdrawn, the patient is given a dry sterile cotton swab to press over the site of vene-puncture. Elbow may be flexed to keep the cotton swab in place till the blood stops. Any blood spill is carefully wiped with 70% ethanol.
- All the swabs and cotton pieces are placed in plastic bags for disposal. If the outside of the vial is visibly contaminated with blood, it should be cleaned with 10% freshly prepared sodium hypochlorite solution.
- The blood is allowed to clot for 30 minutes (not more than 2 hours) at room temperature. The clot may be gently broken if necessary using sterile pasteur pipettes.

8.2 Separation of serum

- The vial is centrifuged at 1200g (3000 rpm) for 10 minutes to separate serum to avoid haemolysis. If no centrifuge is available, the blood with clot may be left in the refrigerator at $+4^0C$ for overnight. The clot will retract and get separated from serum.
- The specimen vial is un-stoppered, the serum is drawn off by sterile pasteur pipette and transferred to a sterile plastic screw capped leak proof tube.

8.3 Addition of preservative

- Preservatives should not be added since it inactivates conjugates and gives rise to false serological results.

- If necessary, 5 bromo, 5 nitro, 1-3 dioxane in propylene glycol at a final concentration of 0.05% is recommended as preservative.
- Thiomersal at a final concentration of 0.01% is effective only for a few weeks as it loses activity when exposed to light.

8.4 Storage of serum specimens

- The sera samples are placed in leak proof plastic containers in the refrigerator at $+4^0$C, for storage.
- The outside of the container is checked for visible contamination with blood which should be cleaned as in 8.1.
- Then the specimen vials are packed in a second tightly capped unbreakable container surrounded by adequate packing material.
- For storage for a long time, deep-freezing at -70^0C is advised.

8.5 Transport of serum samples

- The specimen tube in which serum is to be transported, should not have crack or leakage. Preferably, it should be made of plastic and should be screw capped. The outside of the container is checked for any visible contamination with blood which should be disinfected as in 8.1.
- The tube is labelled and then placed in a second tightly capped unbreakable container (Fig. 8.1) surrounded by adequate packing material (like tissue paper, absorbent cotton etc.) to absorb liquid, if leakage occurs accidentally.
- The secondary container should also have a label. This is placed in a thermocol box having ice packs to maintain proper cold chain system during transit.
- A proforma with details i.e. name, age sex. risk factors, history of previous testing etc. should accompany the specimen.
- A biohazard symbol (Fig. 8.2) must be fixed outside the thermocol box. This box can now be sent to a distant laboratory.

8.6 Transport of whole blood for virus isolation and CD4, CD8 studies

The universal precautions as mentioned above should be followed :

- Blood is collected in heparinized/EDTA screw capped vials.
- It is then placed in a second unbreakable container with adequate packing material.
- It should be transported in wet ice so as to reach the laboratory within 24 hours.
- For virus isolation, if the specimen cannot be transported immediately after collection, it should be frozen.

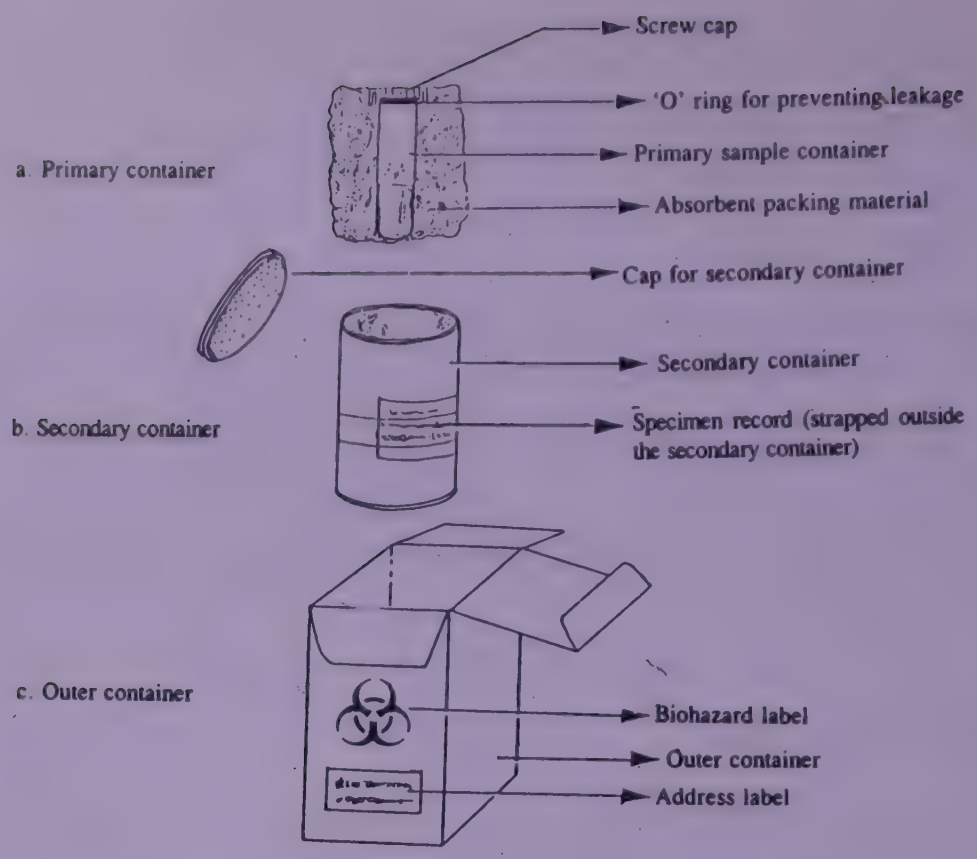


Fig. 8.1 Packing infectious substances for the post

Fig. 8.2 Infectious substance label

SECTION –II : PARASITIC INFECTIONS

Chapter –9

PARASITIC INFECTIONS OF THE GASTROINTESTINAL TRACT ASSOCIATED WITH HIV/AIDS

9.1 Introduction

As such, parasitic diarrhoeas are an important cause of morbidity in developing countries. Some of the parasites are well established enteric pathogens e.g. Entamoeba histolytica, Giardia lamblia, and Balantidium coli etc. and others are opportunistic pathogens e.g. Cryptosporidium, Isospora, Microsporidium and Cyclospora etc. Only a small percent of individuals harbouring the established enteric pathogenic parasites suffer from symptomatic disease in an immunocompetent host. However, with the advent of HIV/AIDS, the scenario has changed. Chronic, recurrent infections with all the enteric parasites have been reported from all over the world with varying frequencies. The rate of infection with a particular enteric parasite in HIV/AIDS patient will depend upon the endemicity of that particular parasite in the community.

The progressive destruction of the immune system by chronic HIV infection leading to progressive fall in level of CD4 cells (<200 to <50) is responsible for the occurrence of infections by opportunistic micro-organisms as also the recurrent, prolonged, intractable and severe nature of infections with all types of micro-organisms. It has been estimated that the probability of developing a serious infection is 33% at 1 year and 58% at two years, if CD4 counts are below 200.

Chronic parasitic gastrointestinal infections mainly diarrhoea have been reported in 26% to 66% of patients with AIDS in North America and Europe (developed countries) and in 63% to 93% of patients in Haiti and Africa (developing countries). Protozoa are the most common cause particularly in developing countries. Most of the opportunistic enteric protozoa have gained importance during recent times on account of their association with HIV/AIDS[1]

The spectrum of diarrhoeal diseases due to enteric parasites (established pathogens as well as opportunistic pathogens) is still evolving. However, infections with G. lamblia and Cryptosporidium have been reported from patients with AIDS in India.

Most common enteric opportunistic parasites which have been associated with HIV/AIDS include [1-7]:

- Cryptosporidium spp.
- Isospora belli
- Cyclospora spp.
- Microsporidium spp
- Strongyloides stercoralis
- Giardia lamblia
- Entamoeba histolytica

(Fig. 9.1 to 9.11 depict the oocysts, cysts and trophozoites etc. of parasites encountered in faeces)

Table -9.1 Systems involved and clinical presentation in HIV infected [1-7]

Parasite prevalence	System involved	Clinical presentation	Symptoms	Prevalence developed -developing countries
Cryptosporidium (30% to 50%)	Small intestines Billary system (10%-15%)	Asymptomatic carriage of oocysts to life threatening enteritis complicated by biliary tract involvement	Epigastric pain with nausea, flatulence malaise and chronic diarrhoea leading to weight loss	8% upto 50%
Isospora	Small intestines Lymphnodes: Mesenteric Periaortic Mediastinal (detected early at biopsy)	Cramping abdominal pain anorexia weight loss, low grade fever (±)	Watery diarrhoea, without blood and leukocytes	1%-3%, 8% -20%
Cyclospora	Small intestines	Anorexia with extreme fatigue, abdominal pain bloating, flatulence, fever and weight loss	-do-	Presence of parasite has been reported from all over except USA and Western Europe. Prevalence of infection by this parasite is not knonw
Microsporidia (Enterocytozoan bieneusi)	Small intestines Biliary system	Predefaecation abdominal pain, anorexia, nausea	Chronic or intermittent watery diarrhoea without blood and mucous, cholangitis weight loss	23% to 33% (From all over)
Giardia lamblia	Small intestines Biliary system	Similar to Cryptosporidium	-do-	Not exactly known on account of these parasites being well known pathogen
Strongyloides stercoralis	Small intestines		-do-	

Characteristics of intestinal spore forming protozoa

- All have been identified relatively recently.
- Life cycle is similar.
- Common in tropics with poor sanitation.
- Transmission is faecal-oral through contaminated water, food and person to person contact.
- Cause travellers diarrhoea, infections in children and outbreaks in homes/institutions.
- Mechanism of pathogenesis is not exactly known. Intense infection of small bowel associated with inflammatory infiltrate may be responsible for abnormalities of absorption, secretion and motility of intestines. Usually infections are nonulcerative and noninvasive.
- Asymptomatic infections are common. Self limited diarrhoea mostly occurs in healthy individuals, sometimes there may be prolonged diarrhoea.Chronic diarrhoea occurs in immunodeficient persons.
- Diagnosis is by microscopic examination of stool and demonstration of typical oocysts
- Antibiotic treatment is indicated in immunodeficient persons.

Table -9.2 Diagnostic characterstics of parasitic causes of diarrhoea in HIV/AIDS [1 to 7]

Parasite	Size (im)	Stage	Specimen	Diagnostic procedure	Comments
Cryptosporidium spp.	4–6	Oocysts with 4 crescentic sporozoites	Stool	– Wet preparation – Direct and after concentration – Modified acid fast stain – mAB based assays (ELISA and IF)	– Concentration method is better, modified acid fast stain gives results.oocysts stain pink.
Isospora belli	20–30 by 10–19	Oocysts with 4 sporozoites in 2 sporocysts	Stool Intestinal biopsy	– Wet preparation – Modified acid fast stain – Histology – EM	– Concentration method is better intestinal biopsy may be positive and stool may be negative – Spindle shape spore with 2 sporocysts pink in colour in acid fast stain
Microsporidia (Enterocytozoan bieneusi)	1–5 by 2–7	Spore	Intestinal or tissue biopsy and touch preparation Stool	– Histology – EM – Giemsa stain – Modified trichrome stain – IF – EM	– Identification difficult – EM–better – Modified trichrome and IF stains are better
Cyclospora	8–10	Occyst with 2 sporozoites in 2 sporocysts	Stool Intestinal biopsy	– Wet preparation – Modified acid fast stain – Histology – EM	– Seen well in net preparation – Also in acid fast stain
Giardia lamblia	8–12 by 7–10 9–21 by 5–15	Cyst Trophozoite	Stool Duodenal aspirate	– Wet preparation – Giemsa stain – ELISA to detect antigen	– Concentration technique may be better – ELISA more sensitive
Blastocystis hominis (Fig. 9.7 – Trichrome stain)	5–20	----	Stool	– Wet preparation	– One large vacuole with compressed cytoplasm forming a granular ring which stains pale yellow in iodine preparation
Strongyloides stercoralis	200–300	Larva	Stool Duodenal material	– Wet preparation	– Rhabditiform larva seen very well

Wet preparation = Saline as well as iodine preparation
IF = Immunofluorescent stain, EM = Electron microscopy
ELISA = Enzyme linked immuno-assay, mAb = Monoclonal antibodies
Ag = Antigen

Table –9.3 Morphologic and other characteristics of spore/oocyst forming protozoa.

Characteristic	Cryptosporidium	Isospora	Cyclospora	Microsporidia
Oocyst	4–6 microns with 4 crescentic sporozoites	20–30 microns with 4 sporozoites in 2 sporocysts	8–10 microns with 2 sporozoites in 2 sporocysts	1–2 microns by upto 4 microns
Wet Preparation	Not seen well	Seen well	Seen well	Hard to differentiate from bacteria and debris
Modified acid fast stain	Seen well red in colour (Fig. 9.1)	Red, seen well (Fig. 9.2)	Variable – not easy (Fig. 9.3)	Variable not easy (Fig. 9.4)
Trichrome IFA stain	– Seen well	– –	– –	Best seen with modified trichrome and fluorescent stains (Fig. 9.6)
Morphology in small bowel biopsy specimen	Easily seen as 4 micron round blue dots on the apical membrane of the enterocyte	Seen as 20 micron oval blue enterocyte inclusions	Has only been seen with electron microscopy	2–3 micron apical inclusions in the enterocyte
Travellers diarrhoea	Common	Less common	Common	Rare
Chronic diarrhoea in AIDS patients	Common	Common	Common	Common

9.2 Collection of specimen

Specimen – Stool/duodenal aspirate

(Intestinal biospy is not feasible in India, presently)

Adequate amount (4 ml/4 gm) of specimen should be collected in clean dry container (Plastic/ tin box with lid etc). Stool specimen should be examined as such and also after formol–ether concentration technique.

Preservation for transport

Requirements

- Formaldehyde – 100 ml
- Sodium chloride solution 0.85% – 900 ml

Add 100 ml of formaldehyde to 900 ml of 0.85% saline and mix. The solution can be used for preservation of stool specimens.

9.3 Laboratory diagnosis

9.3.1 Wet Slide preparation

Requirements

- Microscopic slides
- Coverslips (20 mm by 20 mm)
- Grease pencils
- Wooden applicators (broom-sticks)
- Sodium chloride solution (8.5 g Sodium chloride in 1 litre distilled water).
- Lugol's iodine (diluted 5 times).

Lugol's iodine

Requirements

Iodine	1 g
Potassium Iodide (K1)	2 g
Distilled water	to make 100 ml

To the ground iodine and potassium iodide(KI)mixture add water, a few ml at a time, each time thoroughly grinding the mixture till iodine and K1 are completely dissolved. Put the solution in dark coloured glass bottle(brown) and make up the volume to 100 ml.

Procedure

- Put 1 drop of Sodium chloride solution in the middle of left half of slide and one drop of iodine stain in the middle of the right half of the slide.
- Using a wooden applicator take a small portion of stool from the middle and surface of the specimen including mucous, blood stained mucous. If the stool is liquid, take a portion from the surface.
- Mix the sample with a drop of Sodium chloride.
- Mix the second sample with a drop of iodine solution.
- Place one coverslip over each drop in such a way that the two drops do not mix.
- Mark the number of specimen on slide with grease pencil.

Examine the preparations under x10 and x40 objectives for cysts, larvae, spores and ova, most of which will appear colourless in saline and yellowish brownish in iodine preparations.

9.3.2 Formol-ether concentration technique

Requirements

Basic glasswares		
Formaldehyde	10 ml.	mix first three to make
NaCl	0.85 gm	formal - saline 10%
Distilled water	100 ml	
Solvent ether		

Procedure

- 1 gm of stool is mixed with 10 ml of distilled water in a graduated centrifuge tube.
- The suspension is then strained through a double layered piece of gauze to remove the coarse particles.
- The filtrate is centrifuged at 2000 r.p.m.
- The supernatant fluid is discarded and the sediment is suspended in 3–4 ml of 10% formal saline.
- To this equal amount of solvent ether is added and the mixture is mixed on a whirl mixer manually and centrifuged again at 2000 r.p.m. for 2 minutes.
- Fatty debris formed at the junction of two fluids is broken with a stick and the supernatant discarded.
- A drop from the sediment suspension is mixed with one drop of 5% iodine and another drop of suspension is placed on a clean glass slide without adding iodine.
- A thin coverslip is placed on each drop and examined for cysts and ova of parasites.
- Faecal smear can also be made for acid fast staining, trichrome staining and may be fluorescent antibody staining.

9.3.3 Kinyoun acid fast staining of faecal smear for oocysts/spores of protozoan parasites

Requirements

- Carbol fuchsin

  Solution A : Basic fuchsin 3 gm/100 ml absolute ethanol (95%)

  Solution B: 5% aqueous phenol.
- Decolourising solution Conc.HCl 3 ml + 95% ethanol 97 ml
- Methylene blue 0.3%

Procedure

- Prepare the smear by the formol ether concentration technique.
- Fix the smear by passing over flame.
- Cover the smear with carbol–fuchsin.
- Keep for 30 minutes and wash with water.
- Pour decolorising solution and keep for 20 minutes.
- Wash gently with water.
- Counterstain with 0.3% methylene blue for 30 seconds.
- Wash gently in running water and air–dry
- Examine under high power and oil immersion lens for pink to deep red oocysts (Cryptosporidium,Isospora, Cyclospora and Microsporidia as described) against a blue background.
- Alternately modified ZN stain can also be used.

9.3.4 Modified trichrome staining (for spore forming enteric protozoa particularly Microsporidia).[4]

Requirements

- Basic glassware (slides, coverslips, pipettes etc.)
- 10% formalin solution
- Glacial acetic acid
- Absolute ethyl alcohol (to be made into 90% and 95% for use)
- Methanol
- Acid - alcohol (4.5 ml acetic acid + 995.5 ml. of 90% ethyl alcohol)

Modified trichrome stain

- Chromotrope 2 R	6 g
- Fast green	0.15 g
- Phosphotungstic acid	0.7 g

These ingredients are made to stand for 30 minutes in 3 ml of glacial acetic acid and are then mixed with 100 ml of distilled water.

Procedure

- The method is used for detecting Microsporidia by light microscopy.
- 10μl aliquots of a suspension of unconcentrated liquid stool mixed in 10% formalin (1.3 ratio).
- Spread over an area 45 by 25 mm on the microscopic slide.
- Smear is dried and fixed in methanol for 5 minutes.
- Stain for 90 minutes with the modified trichrome stain (chromotrope based).
- Rinse in acid alcohol for 10 seconds and then briefly in 95% alcohol.
- Dehydrate the smear successively as below :
  - 95% alcohol for 5 minutes
  - 100% alcohol for 10 minutes
  - Hemo-De (Xylene) for 10 minutes
  - Examine slide under oil immersion lens

The Microsporidium spores appear ovoid, refractile, 1.5 by 0.9 ìm, with the spore wall stained bright pinkish red against light greenish background. Some of the spores appear acellular, others show a distinct pinkish-red-stained belt like stripe that girds the spore diagonally or equatorially. Sometimes yeast and bacteria may also appear red in colour but these can be identified by their shape, size and solid staining character. Smears prepared after various concentration techniques do not give good results.

9.3.5 Detection of Cryptosporidium sp. oocysts and/or G.lamblia by immunofluorescence. Follow the instructions of the manufacturers of the kit for performing this technique.

Requirements

- Multispot microscope slides and coverslips (22 x 64 mm)
- Acetone
- Adjustable automatic pipette (5-50 ul), and disposable tips
- FITC labelled anti-Cryptosporidium Mab (diluted optimally) and/or G.lamblia antibody or both in the same kit.
- Humidified chamber
- Glycerol-based mounting medium as suplied with the kit
- Flourescence microscope with FITC filters and x 20 or 25 x 40, and x 100 objective lenses suitable for fluorescence
- Photobleaching inhibitor such as Goitfluor (City University, Department of Chemistry, UK).
- 30^0 C incubator. Oocysts and/or Giardia cysts positive and negative controls

Method

- Pipette 25 μl of emulsified stool or formol ether concentrate on to and spread over a well of the multispot slide.
- Air dry the slide at room temperature.
- Fix the slide for 1-5 min in acetone and air dry.
- Place 25 ul of the FITC labelled monoclonal antibody (at the predetermined working dilution) on to the fixed air dried specimen and the oocyst/cysts positive and negative controls.
- Incubate the slide (s) horizontally in the dark in a humidified chamber for 30 min at 37°C.
- Rinse the slide in a gentle stream of water (e.g. with a wash bottle).
- Drain the excess moisture from the slide and add two or three drops of the mounting medium supplied.
- Apply a coverslip to the slide ensuring that no air bubbles are trapped over the specimen.
- Examine the negative and positive controls and record your findings.
- Examine the test well for fluorescing oocysts/cysts using the x 20 (for x 25) objective lens. Cover the whole area of the spot with either vertical or horizontal sweeps. Ensure that the whole area is viewed not only oocysts/cysts present.
- Assess the numbers of oocysts present.

Important points

- Do not press down on the coverslip, but allow its weight to displace the mounting medium. Inverting the slide on an absorbent tissue will remove the excess mounting medium.
- Specific fluorescence localized on the oocyst wall, on circular objects measuring 4-6 ìm diameter or pear shaped cysts of Giardia, should be demonstrable in the positive control only. Specific fluorescence is applegreen in colour, background fluorescence is red (due to the

presence of Evans blue which reduces non-specific fluorescence). Non-specific fluorescence is yellow. Always refer to the positive control to ensure that the size, shape, and colour of the putative oocyst/cysts are consistent with those of the positive control.

Numbers can be recorded as scanty, moderate, or numerous.

9.4 References :

1. Goodgame, R.W. understanding intestinal.spore forming protozoa : Cryptosporidia, Microsporidia, Isospora and Cyclospora. Ann. Int. Med. 1996 : 124 : 429-441.

2. Weber, R. Bryan, R.T. Microsporidial infections in immunodeficient and immunocompetent patients. Clin. Infect. Dis. 1994 : 19 : 517-521.

3. Ooi. W.W., Zimmerman, S K, Needham, C.A. Cyclospora spp. as a gastrointestinal pathogen in immunocompetent hosts. J. Clin. Microbiol. 1995 : 33 : 1267-1269.

4. Weber, R., Ralph., T.B., Rober. L. et al. Improved light microscopical detection of Microsporidia spores in stool and duodenal aspirates. New Eng. J. Med. 1992 : 326 (3) : 161-166.

5. Mannheimer, S.B. and Soave, Rosemary. Protozoal infections in patients with AIDS. Infect. Dis. Clin. North. America. 1994 : 8 : 483-498.

6. Ernest, N.G., Markell, E.K., Fleming, R.L. and Fried, M. Demonstration of Isospora belli by Acid Fast Stain in patients with AIDS. J. clin. Microbiol 1984 : 20 : 384-386.

7. De-Hovitz, J.A., Pape, J.W, Boncy, M et al. Clinical manifestation and therapy of 1. belli infection in patients with AIDS. The New Eng. J. Med. 1986 : 315 (2) : 87-90.

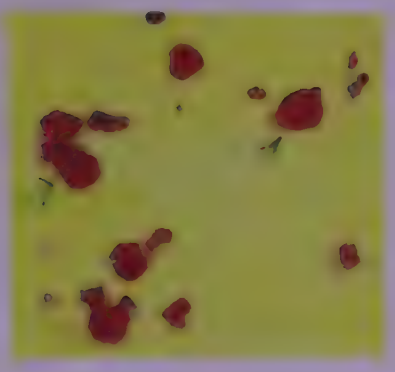


Fig. 9.1
CRYPTOSPORIDIUM OOCYTS
FAECAL SMEAR AFTER
CONGENTRATION
MODIFIED AF STAIN
X 1000

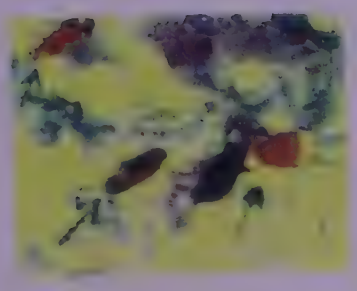


Fig. 9.2
ISOSPORA BELLI OOCYSTS
FAECAL SMEAR AFTER CONCENTRATION
MODIFIED ACID FAST STAIN X 400

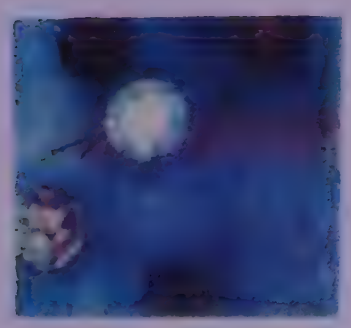


Fig. 9.3
CYCLOSPORA OOCYSTS
FAECAL SMEAR AFTER
CONCENTRATION
MODIFIED AFS X 1000

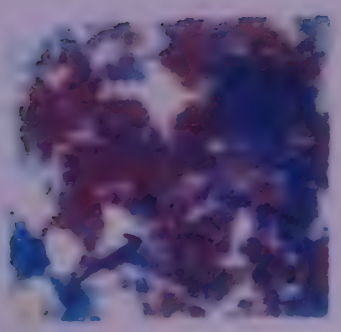


Fig. 9.4
MICROSPORIDIAL SPORES
DUODENAL ASPIRATE
ACID FAST STAIN X 1200

ACID FAST STAINING FOR PROTOZOAL OOCYSTS AND SPORES

Fig. 9.5
E.HISTOLYTICA TROPHOZOITE WITH 2 INGESTED RBCs FAECAL SMEAR TRICHROME STAIN X 1000

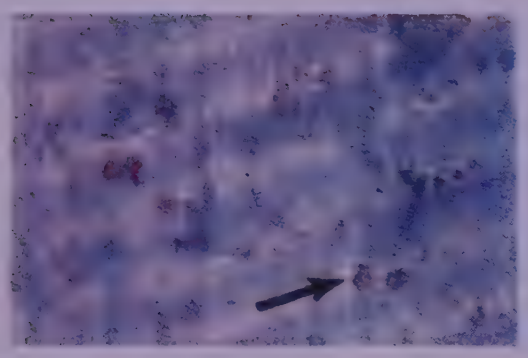


Fig. 9.6
MICROSPORIDIAL SPORES FAECAL SMEAR AFTER CONCENTRATION TRICHROME STAIN X 1200

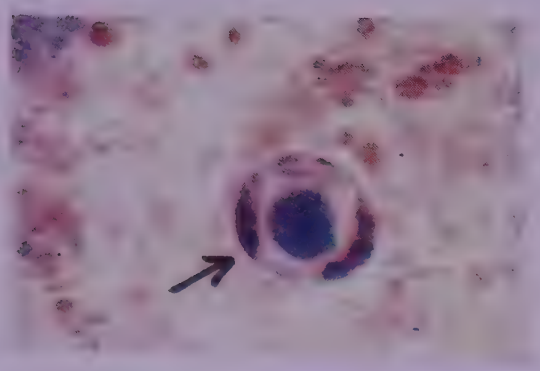


Fig. 9.7
BLASTOCYSTIS HOMINIS FAECAL SMEAR TRICHROME STAIN X 1000

TRICHROME STAINING FOR BETTER DEMONSTRATION OF PROTOZOAN PARASITES

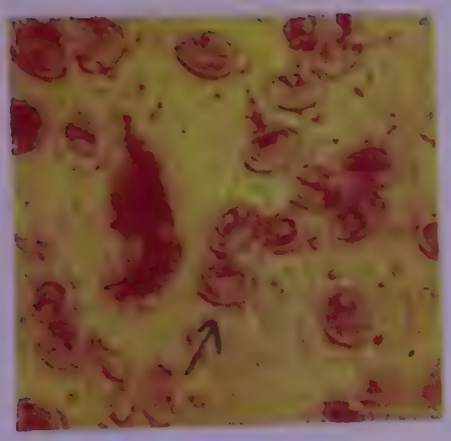


Fig 9.8
GIARDIA LAMBLIA CYSTS
FAECAL SMEAR AFTER CONCENTRATION,
GIEMSA

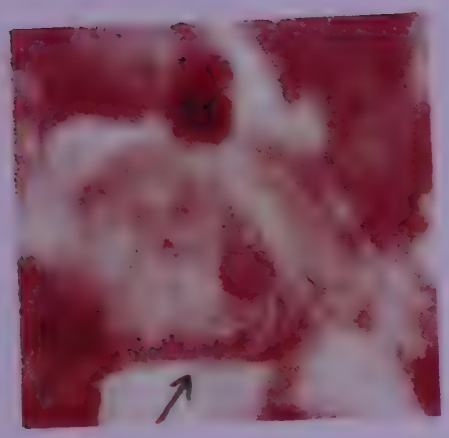


Fig. 9.9
E. HISTOLYTICA TROPHOZOITE
FAECAL MUCOUS SMEAR
GIEMSA

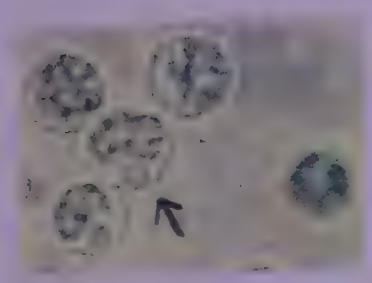


Fig. 9.10
CYCLOSPORA OOCYSTS
FAECAL WET SMEAR
AFTER CONCENTRATION

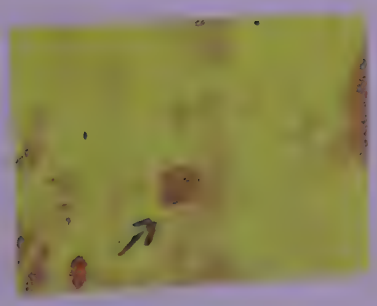


Fig. 9.11
ISOSPORA BELLI
FAECAL SMEAR
IODINE PREPARATION

Chapter – 10

HIV AND TOXOPLASMOSIS

10.1 Introduction

Toxoplasmosis is a world-wide zoonotic infection which is caused by the protozoan parasite Toxoplasma gondii; it produces a generalised disease but the central nervous system frequently bears the major brunt.

Many other animals in addition to man are affected. The domestic cat and other felines constitute the definitive host. The life-cycle usually involves the cat, together with several small rodents and birds (the intermediate host); man and other large mammals may suffer serious disease but they play no part in the cycle. Transmission to humans usually occurs in one of two ways – by the oral route, primarily through eating food and by vertical transmission from mother to foetus during an acute infection. Rarer forms of transmission are via organ transplantation and granulocyte transfusion.

Toxoplasma gondii is now recognised as a major cause of neurologic morbidity and mortality in patients with acquired immunodeficiency syndrome (AIDS). Like other opportunistic pathogens, T.gondii causes asymptomatic or mildly sysmptomatic infections in normal hosts, but rapidly progressive, fatal disease in immunosuppressed patients. Most patients with advanced HIV disease respond to therapy for toxoplasmosis hence prompt diagnosis and treatment are crucial.

10.2 Magnitude of problem

The specific prevalence of toxoplasmosis in patients with AIDS is unknown. The figures available are almost certainly an underestimate because patients are reported at the time of diagnosis of AIDS, and undoubtedly additional patients develop toxoplasmosis during the course of their illness. In the United States, it develops in 3–10% of patients with AIDS. In Europe and Africa, where the overall seroprevalence of toxoplasma is higher, it is estimated that toxoplasmic encephalitis will ultimately develop in 25 to 50% of patients with AIDS.

In some of the countries in Asia Pacific Region the percentage of toxoplasmosis in patients with AIDS has been found to be 11.3% in Australia, 5.4% in India, 18.6% in Indonesia, 1.8% in Japan and 4.5% in Thailand.

10.3 Clinical presentation

The clinical manifestations of active toxoplasmosis in AIDS patients are varied, but the majority of patients have symptoms referable to a focal process in the central nervous system, Symptoms usually develop subacutely, over the course of a few weeks. The most common presenting symptoms include focal neurologic abnormalities, most often mild to severe hemiparesis, confusion and lethargy. Encephalopathy and focal abnormalities will frequently occur in the same patient. focal or generalised seizures are present in 15% to 43% of patients. Headaches, which are often severe, bilateral and unremitting, occur in approximately half the patients. Fever occurs in about 60% of patients. signs of meningeal irritation are rare, and have been reported in less than 10% of patients.

Involvement of other organ systems such as lungs, heart, muscles, gastro intestinal tract is commonly seen at autopsy, but is generally asymptomatic during the life. Rarely, patients will present with severe symptomatic involvement of one or more of these organ systems.

Organ system involvement in AIDS patients with Toxoplasmosis is given below :

Frequent

Brain (focal encephalitis)

Rare

Eye (retinochoroiditis)
Lungs
Testicles

Rare of uncertain clinical significance

Heart
Stomach
Adrenals
Pancreas
Striated muscle

10.4 Laboratory diagnosis

To effectively diagnose toxoplasmosis in patients with advanced HIV disease, physician must (I) be aware of the disease and its subtle presentations, (2) understand the limitations of radiographic and serologic tests in diagnosing toxoplasmosis in patients with advanced HIV disease (3) initiate empiric therapy for suspected CNS toxoplasmosis when appropriate and (4) use invasive procedures when necessary.

10.4.1 Collection and transportation of specimen

Because of the poor viability of Toxoplasma outside the host and its somewhat fragile nature, it is important that specimens for culture or histology be obtained and transported to laboratory as quickly as possible. Tissue specimen should be neither frozen nor allowed to desiccate. If there is delay in transportation, keep in refrigerator at + 4^0 C. The diagnosis of toxoplasmosis consists of demonstration and isolation of the organism and demonstration of specific antibodies in the serum.

For the above tests following clinical specimens have to be collected.

- Enlarged lymph node, brain biopsy, lung biopsy, CSF, or bone-narrow aspirate for histopathology and culture.
- Whole blood or CSF in acute phase for culture.
- Blood in plain vial for antibody detection. It should be allowed to clot and serum is separated by centrifugation.

10.4.2 Demonstration and isolation of the parasite

The optimal method for diagnosing toxoplasmosis in any patients is by the demonstration of the tachyzoites in clinical or pathological specimens or by culturing the organism from clinical specimens.

Demonstration of the parasite

The most common method used currently is histopathologic detection of organisms in biopsy specimens. Because brain is the organ most frequently affected, obtaining a biopsy specimen is somewhat more complicated than at other sites. The development of newer techniques for brain biopsy, specifically the use of CT scan directed stereotactic needle biopsy, has improved the safety of such procedures.

Toxoplasma gondii can be detected in biopsy specimens by a number of techniques.

Haematoxylin and eosin (H&E) staining

The organism, specifically the cyst form, may be recognised in H & E stained specimens, although the tachyzoites may be much more difficult to discern clearly. Tissue sections stained with haematoxylin and eosin reveal ovoid or rounded tachyzoites (3x4μm) or bradyzoites (which are smaller in size but more closely packed). Because of the necrosis and other factors, the organism may not be seen in routine H and E sections in over 50% of cases.

Giemsa staining

(i) Fix the smear with pure methyl alcohol or ethyl alcohol for 3 o 5 minutes and allow it to dry.

(ii) Prepare 5% solution of Giemsa stain with tap water and pour it over the film and keep it for 60 minutes.

(iii) Then flush the slide in a gentle flow of tap water.

(iv) Examine the stained film under oil immersion lens. Trophozoites of toxoplasma are seen. Due to sparse distribution it is usually difficult to find organisms by this method.

Fig. 10.1 to 10.3 show the trophozoites of T.gondii from culture, tissue and from exudate

Flourescent antibody staining

Immunofluorescence, using monoclonal antibodies against T.gondii to detect the organism in impression smears made from biospy specimens, has also been found to be useful in establishing the diagnosis. Indirect fluorescent antibody staining technique using anti-human gammaglobulins is also being used.

10.5 Isolation of the parasite

Mice inoculation

Tissue samples are ground with physiologic saline in a mortar and pestle and inoculated intraperitoneally in young albino mice. Whole blood or cerebrospinal fluid can also be inocualted When biospy material is contaminated it can be treated with antibiotics prior to inoculation.

When toxoplasma are present the animals show the presence of periotoneal exudate in 7-10 days and trophozoites can be readily identified by examination of the fluid under the microscope.

Several blind passages in mice may be required before the organism becomes apparent.

Mice surviving more than 4 weeks should be tested for antibody and, if positive, should undergo necropsy to demonstrate the organism in the tissues using Giemsa stain.

Tissue culture

More recently tissue cultures have also been used for isolation of the parasite. This is generally considered a less reliable procedure. Acute reactivated toxoplasmosis has been diagnosed by inoculation of tissue culture with sera from HIV-infected patients with CNS, pulmonary and ocular toxoplasmosis. This technique cannot be used in routine because it is time consuming, cumbersome and expensive. This may be helpful, however, in identifying toxoplasmosis in selected patients whose clinical pictures, serologies, and biopsy results are non diagnostic.

Specific antigen detection

Techniques for detecting specific toxoplasma antigen in tissues and body fluids are important in determining acute infection. A peroxidase - antiperoxidase technique has been used to identify antigen and whole tachyzoites in tissues. An Enzyme linked Immunosorbent Assay has been developed and has been found to be sensitive and specific for detecting antigenaemia.

10.6 Serological diagnosis

The variety of serologic responses among HIV infected persons with toxoplasmosis reflects the different forms of this disease in various parts of the world. In North America, where the vast majority of toxoplasmosis cases in persons with HIV disease are due to reactivation of latent infection, most of the patients have detectable serum IgG titers. While the presence or absence of IgG titers is important in this population, the actual level of IgG titers cannot be used to identify patients with active as opposed to latent disease since low titres are common in immuno compromised patients with active disease. In general, patients with active CNS disease tend to have higher titers than those with asymptomatic, latent infection, but this is not sufficiently reliable to be used diagnostically. Furthermore, reactivation is not reliably associated with a rise in serum IgG antibody titre. Unfortunately serum IgM titers are variable and unreliable in patients with HIV disease.

Despite the numerous limitations, serum toxoplasma serologic studies play a useful role in management of HIV infected patients.

IgG titer should be measured for all HIV infected patients early in their disease since a positive serologic finding, regardless of the titer indicates the patient is at risk for reactivation of latent toxoplasmosis.

In patients who present with signs and symptoms suggestive of reactivated toxoplasmosis, an IgG measurement alone cannot prove or disprove the diagnosis. A negative IgG titre is unusual in a patient with reactivated toxoplasmosis and should make the clinician give more serious consideration to alternative diagnosis.

In countries like France, where upto 20% of CNS toxoplasmosis in patients with advanced HIV disease is due to acute disease, IgM anti-toxoplasma antibodies are more useful and should be evaluated.

Tests

Sabin-Feldman dye test (SFDT)

This is the first successful quantitative serological procedure for toxoplasmosis, is now performed only in specialised laboratories and has for the most part been superseded by newer methods. The test is highly specific and sensitive.

In this test, live tachyzoites are incubated with the patient's serum. If specific IgG antibody is present, the tachyzoites will lyse and not stain when methylene blue is added. The test titer is reported as the dilution of serum at which half of the organisms present are stained. The measured IgG antibody appears 1 to 2 weeks after infection or reactivation, and peaks at 6–8 weeks. Titers gradually decline over 2 to 3 years, but may persist at low levels for life. The drawbacks of the test are need for specialised training and special reagents including live Toxoplasma organism.

Indirect fluorescent antibody test

The IFA is the most widely available serologic test for toxoplasmosis and measures the same IgG antibody as the SFDT. IFA test has three major advantages over the dye test : it uses a more or less permanent antigen or formalin killed Toxoplasma organisms fixed on a slide; all special reagents are commercially available; and both IgG and IgM antibodies can be measured independently. Its only major disadvantage compared with the dye test is the requirement for a fluorescent microscope with an ultraviolet light source. The test is considered to be sensitive, accurate and reproducible. For performing the test, following procedure can be used.

(i) Coat the multispot slides (degreased with acetone) with the antigen (Tachyzoites forms of Toxoplasma gondii) and fix with chilled acetone after they are air dried. Place the slides in a desiccator and keep at -20^0C until used.

(ii) Dilute the patients serum as 1:8 with phosphate buffered saline (pH range is 7.2 - 7.6) and make two fold dilution upto 1:4096.

(iii) Transfer approximately 10 μl diluted patient serum in the respective wells of the coated multispot slide along with positive and negative controls on each test slide. Incubate the slides in moist chamber at RT for 60 minutes.

(iv) Wash three time with PBS, 5 minutes each wash.

(v) Cover the slides with FITC conjugate (Sigma F -6380) suitably diluted (dilution already worked out) and incubate in moist chamber for 60 minutes at room temperature.

(vi) Wash the slides twice with PBS and once with distilled water, 5 minutes each wash.

(vii) Mount the slides with mounting fluid (90% glycerol PBS buffer) pH being slightly alkaline and examine under fluoroscent microscope using an appropriate reference filter.

Enzyme Immunoassay (EIA)

EIA has been developed for detection of both IgG and IgM antibodies. The major advantages of EIA are that soluble antigen can be used and that the tests tend to be more sensitive than IFA. EIA correlates well with IFA, but is technically much more difficult to perform and should be monitored by a more stringent quality control system. For detection of IgM antibodies, a double sandwich immunoglobulin - M enzyme linked immunosorbent assay (DS-IgM-ELISA) has been developed.

EIA for detection of IgG antibodies can be performed as per the following procedure.

(i) Recharge the ELISA plate by filling up the wells with luke warm distilled water at least for 15 minutes. Discard the water by inverting and shaking.

(ii) Prepare predetermined antigen dilution in carbonate bicarbonate buffer pH 9.6 of 0.05 molarity. Add 100 μl of diluted antigen to all the wells and incubate overnight in a refrigerator.

(iii) Discard the antigen and wash the plate three times with phosphate buffered saline (PBS) pH 7.2 - 7.6 containing 1.0% Tween 20. Empty the plate by inverting and shaking.

(iv) Prepared 1.0% skimmed milk solution in PBST and add 400 μl to each of the well and incubate the plate at 37°C for 45 minutes.

(v) Discard the material of the plate and give three washings as in step 3.

(vi) Prepare 1:100 dilution of the test sera samples of well as of positive and negative controls in PBS containing 1.0% BSA. Add 100 μl of test samples as well as positive and negative controls in duplicate to the respective wells and incubate at RT for 30 minutes.

(vii) Wash five times with PBST (PBS with tween 20) and empty as in step 5.

(viii) Add 100 μl optimally diluted HRPO antihuman IgG specific conjugate (A 6029, Sigma Chemicals) prepared in PBST to each well and incubate for 30 minutes at RT.

(ix) Wash again as in step 7.

(x) Prepare substrate solution by dissolving 10 ml. OPD (0rthophenylene dihydrochloride) in 25.0 ml oxide buffer pH 5.0 (citric acid + disodium hydrogen phosphate or K_2HPO_4) and add 25 ml of H_2O_2. Add 100 μl substrate solution to each well and incubate at RT for 10 - 15 minutes depending upon the colour in ten minutes of positive and negative controls.

(xi) Stop the reaction by adding 100 μl of IM H_2SO_4 (1:17 DW) in each well and read the absorbance at 492 nm as reference filter.

Agglutination tests

Agglutination tests like Direct Agglutination Test (DAT) and Latex Agglutination are simple to perform, inexpensive and reasonably rapid but can only be used as screening tests and not as the diagnostic tests. These tests involve the incubation of preserved whole parasites with test serum, specific anti-toxoplasma IgG causes visible agglutination.

Complement fixation and indirect Haemagglutination tests (CFT and IHA)

These tests measure an IgG that appears and peaks later (3-6 weeks after infection) than the antibody measured by the SFDT and the IFA test. If the diagnosis of toxoplasmosis is not considered after the peak of SFDT/IFA has occurred, CFT and IHA may demonstrate a rising titer suggestive of recent infection.

10.7 Molecular techniques

A polymerase chain reaction (PCR) assay for the detection and diagnosis of Toxoplasma infection in human is being developed. The PCR detects extremely small amounts of specific DNA sequences, it is very sensitive and specific for detecting T.gondii in the tissues and body fluids of mice. Studies of PCR on venous blood samples, CSF and brain biopsy specimens of HIV infected suggest that the technique can be used for diagnostic testing.

10.8 References

1. Toxoplasma gondii infection : A feline Zoonosis with potential dangers for the unborn foetus and AIDS sufferer in Parasitic Disease in Clinical Practice G.C. Cook (1990).

2. Toxoplasmosis in patients with the Acquired Immunodeficiency Syndrome. In : Opportunistic Infections in patients with Acquired Immunodeficiency Syndrome. Gifford Leoug & John Mulls, 1989.

3. Toxoplasmosis in the AIDS knowledge base (second edition); P.T. cohen, Merle A. Sande, Paul A Volberdug, 1994.

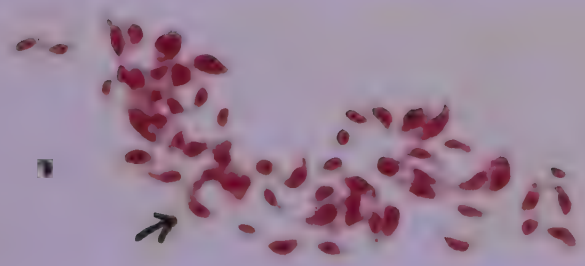


Fig. 10.1 **TOXOPLASMA TACHYZOITES PERITONEAL EXUDATE, MOUSE GIEMSA X 1200**

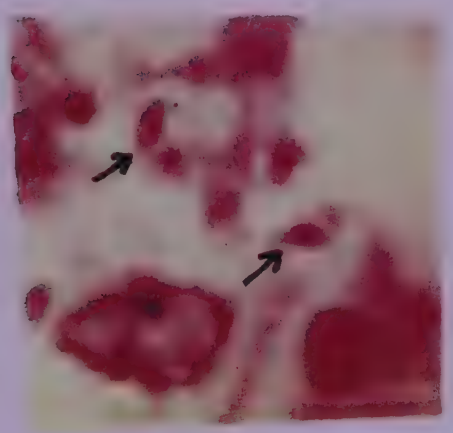


Fig. 10.2 **TOXOPLASMA GONDII TISSUE, GIEMSA X 1200**

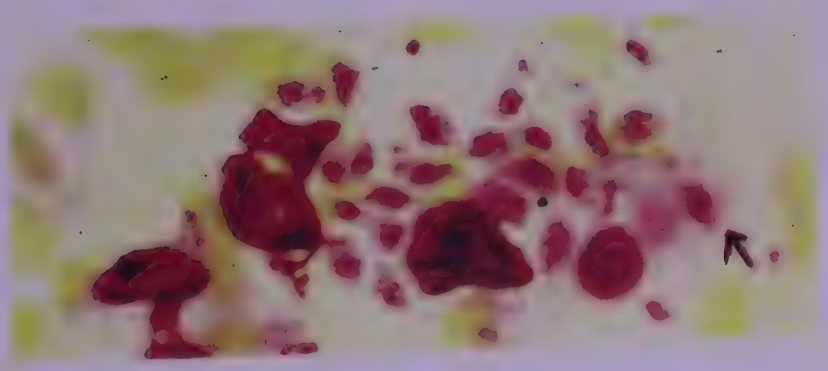


Fig. 10.3 **TOXOPLASMA GONDII LUNG TISSUE, GIEMSA X 1200**

Chapter - 11

HIV AND LEISHMANIASIS

11.1 Introduction

The leishmaniasis are a group of diseases caused by several pathogenic species of the parasitic protozoa Leishmania. They show a variety of clinical presentations including visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL). Most forms or leishmaniasis are primarily zoonotic and all are transmitted by the vector – various species of phlebotomus (female sandfly).

Since 1986, visceral leishmaniasis has been recognised as complication of infection with the human immunodeficiency virus (HIV). It is becoming an important opportunistic infection among persons infected with HIV-1 in geographic areas in which both infections are endemic.

11.2 Magnitude of the problem

Visceral leishmaniasis is thought to represent reactivation of latent infection with Leishmania. The problem is greatest in Southern-Europe, where HIV and L.infantum are both endemic. In Spain, 50 percent of adults with visceral leishmaniasis are HIV positive, and it is estimated that a percent of HIV infected individuals will acquire visceral leishmaniasis. In India, though the exact figures are not available, the cases of visceral leishmaniasis in patients infected with HIV-1 are being reported from different parts of the country.

11.3 Clinical presentation

Clinical leishmaniasis in patients with HIV infection can represent newly acquired or reactivated infection.

The cardinal signs of visceral leishmaniasis in patients with AIDS are unexplained fever, organomegaly, anaemia, or pancytopaenia. Coinfected patients can develop unusual manifestations of visceral leishmaniasis, in part because of atypical localisation of the parasite (e.g. in the gastrointestinal tract). In some cases there may be no fever and 20-40% of the patients have no splenomegaly. Cases of concomitant cutaneous or mucocutaneous leishmaniasis and HIV infection have also been reported. In patients with concomitant HIV and leishmania infection the lesions may also be found in usual sites. Leishmania species normally involve the bone narrow, liver, spleen, lymphnode and jejunal mucosa. In HIV seropositive patients, involvement of oesophageal, gastric, pancreatic, rectal, pulmonary, pleural, cutaneous and hematologic sites has also been noted.

11.4 Laboratory diagnosis

Diagnosis of visceral leishmaniasis should be considered for HIV infected patients who have ever been in leishmaniasis- endemic areas and who have such manifestation as unexplained fever, hepatosplenomegaly or haematological abnormalities in endemic areas. Since this disease shares many symptoms with HIV infection and its complications, and owing to the fact that leishmania antibodies may be absent, the diagnosis can be very difficult and must be ruled out only after performing reliable diagnostic tests such as bone narrow aspiration.

11.4.1 Collection, storage and transportation of specimen

The laboratory results depend directly upon the nature and quality of the specimen received. the collection, transportation and storage of specimens are extremely vital steps in laboratory diagnosis of any disease and hence, must be undertaken with utmost care. The following general rules should be observed while collecting these specimens :

(i) Collect sufficient quantity of specimen

(ii) Avoid contamination by using sterile equipment and aseptic precautions

(iii) Dispatch the specimen immediately to laboratory

(iv) In case the delay is inevitable, keep the specimen at + 4^0 C in a refrigerator

(v) Label all specimens accurately and send all pertinent information to laboratory which will help in better interpretation of the laboratory findings (geographical area, risk group if any for HIV).

Specimen

The diagnosis of leishmaniasis is made by demonstration of or isolation of the parasite from blood or biopsy material and demonstration of Leishmania specific antibodies in the serum. In addition there are a few non specific tests based upon the deviations in the normal blood picture and the serum proteins which may also aid in diagnosis.

For the above tests, the following clinical specimens have to be collected :

(i) Blood (serum)

(ii) Biopsy/aspirate from – bone narrow and/or spleen. Rarely biopsy of lymphnode may also be taken.

(iii) Biopsy from skin lesion.

Blood

It can be collected either through a venepuncture or by finger prick method. By venepuncture 4–5 ml blood should be collected in a plain vial. Allow it to clot and separate the serum.

By finger prick method blood is collected on filter paper. It carries the advantage of easy transportation and requires less storage space. In the laboratory the serum is eluted from filter paper strips by dipping the circle in phosphate buffered saline (pH 7.2) and keeping in refrigerator at $+4^0$ C overnight. Squeeze the circle within the test tube and discard the paper.

Since one circle soaks 0.1 ml of blood and final elution of serum is 20 μl, the final dilution of serum after elution is 1:50.

Bone marrow aspirate

It can be collected from ·

Mid sternal region a little away from the middle line and at the level of second or third intercostal space.

Posterior iliac crest puncture, 1 cm below the posterior superior iliac spine.

It is a safe procedure as compared to the spleen puncture, but is more painful, Its disadvantage over spleen puncture is that when the parasites are scanty, the examination of aspirated material may give negative results.

Spleen puncture

When spleen is considerably enlarged it is one of the most valuable method for establishing the parasitological diagnosis of kala-azar. It requires no special equipment and has proved safe and relatively easy to perform in experienced hands and when proper precautions are taken. The only risk of spleen puncture is that bleeding might continue from the puncture wound in the soft and enlarged spleen, resulting in death. To avoid the risk of heammorhage blood should be examined previously to exclude haemorrhagic diathesis.

Skin biopsy

When skin lesions are present the parasite can be demonstrated in biopsy taken from the lesions.

11.4.2 Demonstration of the parasite

The conclusive evidence in the diagnosis of V L with HIV is the demonstration of the parasite in the specimen. This is achieved by :

(i) Microscopic examination of the stained film

(ii) Culture and isolation of parasite

Direct examination

Giemsa staining of smears. This technique offers clear advantages since a microbiology laboratory is not necessary for the stain, the results are usually available earlier than with the culture method, and diagnosis can be achieved in some cases despite specific treatment having been started. Make smears and stain with Leishman or Giemsa stain. Examine for amastigote forms of the parasite.

Giemsa staining

(i) Fix the film, with pure methyl alcohol or ethyl alcohol for 3 to 5 min and allow it to dry.

(ii) Prepare 5% solution of Giemsa stain with tap water and pour it over the film and keep it for 60 minutes.

(iii) Then flush the slide in a gentle flow of tap water.

(iv) Examine the stained film under oil immersion lens.

Amastigote forms of the parasite (L.D. bodies) can be easily seen (Fig. 11.1 to 11.3).

Leishman staining

(i) Pour Leishman's stain over the dried film and allow it to remain for 30 seconds.

(ii) Dilute the stain with twice its volume of distilled water which should be neutral or slightly alkaline (pH 7-7.2). Prevent drying.

(iii) Allow the diluted stain to remain on the slide for 10 to 15 minutes.

(iv) Hold the slide under an open tap and flush the stain in a gentle flow of water.

(v) Keep the slide in an upright position to drain and dry.

(vi) Examine the dried stained film under oil immersion and look for L.D. bodies.

11.4.3 Culture techniques

A positive culture offers the advantages of an accurate diagnosis and possibly a further characterisation of strains.

Primary isolation from aspirate

(i) Under all aseptic precautions inoculate 1–2 drops of bone marrow or splenic aspirate in culture media like NNN medium, Tobies medium.

(ii) Incubate at 22–25^{0}C for 3–4 days.

(iii) Examine a drop from culture for promastigotes by making a wet smear and its examination under microscope.

(iv) If promastigotes are present, fresh subcultures are made.

(v) Strains can be maintained by doing sub-cultures every 10–15 days.

Primary isolation from blood

The parasite can be isolated from blood in large number of cases. The only disadvantage of this method is that it is slow and takes a long time i.e. about a month.

(i) Under all aseptic precautions collect 1–2 ml of blood and dilute with 10 ml of citrated saline solution (0.85% normal saline containing 2% sodium citrate).

(ii) Allow the cells to settle either by keeping it overnight at 22^0 C in an incubator or by centrifugation.

(iii) Inoculate the cellular deposit in culture media.

(iv) Incubate at 22–25^0 C for 1 to 4 weeks.

(v) At the end of each week examine a drop from culture medium for promastigotes by making a wet smear.

11.4.4. Serodiagnosis

Serological diagnosis is a quick and reliable method of diagnosis as the culture is relatively slow and smears may be negative for the parasite. It consist of demonstration of antibodies. If a patient with HIV also has symptoms of VL, serological tests for leishmaniasis like IFAT and DOT ELISA have good sensitivity.

Serological techniques

Indirect fluorescent antibody test (IFAT)

Principle

Antibody in patient serum can be detected using fluoroscein conjugated anti immunoglobulin. Patient's serum is applied directly to the slides coated with leishmania antigen and than visualised by treatment with fluorescein conjugated anti-immunoglobulin.

Material and equipment required

(i) Multispot slides or simple microscope slides

(fi) Acetone

(iii) Phosphate buffered saline pH 7.2 to 7.4

(iv) 10% glycerol v/v in PBS

(v) Crude promastigote antigen

(vi) Patient's serum and control sera

(vii) Fluorescent microscope

Procedure

(i) Coat multispot slides with crude leishmania promastigote antigen. Fix with chilled acetone. Allow the slides to dry and then store at -20^0 C till used.

(ii) Dilute patient serum 1:50 in phosphate buffer and make doubling dilutions.

(iii) Add 10 μl of patient's serum on antigen coated spot, and keep at room temperature for 60 minutes.

(iv) Wash thrice with phosphate buffered saline for three minutes each.

(v) Add 10 μl conjugated anti human gammaglobulins (suitable dilution) and keep for 30 minutes to one hour

(vi) Wash with phosphate buffer saline for 3 minutes. twice and third time with distilled water.

(vii) Mount with glycerol.

(viii) Apply cover glass and examine under fluorescent microscope.

(ix) Along with each batch put a positive and a negative control serum also.

Interpretation of results

Serum sample showing fluorescence is taken as positive for leishmania antibodies and the highest dilution showing fluorescence is the antibody titre.

Enzyme linked immunosorbent assay (ELISA)

Principle

Patient's serum is added to leishmania antigen bound to microtitre plate. After washing, antibody bound to antigen is revealed by adding any enzyme labelled anti-human immunoglobuln and enzyme substrate.

Material and equipment required

(i) Micro ELISA plate

(ii) Antigen - soluble leishmania promastigote antigen

(iii) 0.05 M carbonate - bicarbonate buffer (pH 9.6) (coating buffer)

(iv) PBS with Tween 20 (washing buffer)

(v) Patient's serum and control serum

(vi) Antihuman horse radish peroxidase immunoglobulin conjugate

(vii) O-phenylenediamine dihydrochloride

(viii) IN Sulphuric acid

(ix) Spectrophotometer or ELISA reader

Procedure

(i) Make predetermined antigen dilution in carbonate biocarbonate buffer. Add 200 il antigen in all the wells. Incubate at 4^0 C overnight.

(ii) Wash with PBS - Tween and fill the wells completely with it.

(iii) Incubate at room temperature for 4–5 minutes and them empty by inverting and shaking.

(iv) Repeat the washing step thrice.

(v) Dilute the test serum in PBS. The optimum dilution must be determinated by checkerboard titration. In our laboratory it is 1: 100.

(vi) Add 200 μl of conjugate diluted in PBS. (Exact dilution already determined).

(vii) Incubate at room temperature for 1 hour.

(viii) Repeat washing procedure as in steps (ii) and (iii).

(ix) While the plate is incubating during washing prepare the substrate in citrate phosphate buffer pH5. This substrate should be made up freshly each time and used immediately. Keep in a dark bottle while it is being pipetted.

(x) Add 200 μl of substrate to each well and leave in the dark at room temperature for 10–15 minutes to develop colour.

(xi) Stop the reaction by adding 50 μl of 1N–sulphuric acid.

(xii) Read the absorbance at 492 nm in a spectrophotometer or ELISA reader.

Interpretation

Visually positive and negative samples can be differentiated by development of brown colour. Use of ELISA reader or spectrophotometer increases the sensitivity of the test and antibody titre can also be assessed.

DOT enzyme linked immunosorbent assay

Principle

Principle is same as that of conventional ELISA test. The only difference is that antigen is coated on nitrocellulose filter discs in this test.

Material and equipment required

(i) Triethanolamine – buffered saline (TBS, pH 7.5)

(ii) Bovine serum albumin, fraction V (BSA)

(iii) 4 Chloro–I–naphthol (4 CIN)

(iv) Horse radish peroxidase (HRPO) conjugated antihuman IgG diluted 1:100 in 1% BSA – TBS (pH 7.4)

(v) Hydrogen peroxide 30%

(vi) Flat bottom 96 well microtitre plates

(vii) Test serum and control serum

(viii) 5 mm Nitrocellulose discs coated with promastigote antigen

(ix) Shaker

Procedure

(i) Put one antigen coated disc in each well.

(ii) Add 75 μl of 5% BSA – TBS.

(iii) Shake microtitre plate on shaker for 1 minute.

(iv) Incubate for 15 minutes at room temperature.

(v) Aspirate off the blocking solution.

(vi) Add 50 μl of serially diluted test sample prepared in 1% BSA – TBS.

(vii) Shake the microtitre plate for one minute.

(viii) Incubate at room temperature for 30 minutes.

(ix) Aspirate off the sera

(x) Wash three times with 100 il of 0.05% (Nonidet) NP 40 – TBS (V/V) with shaking during each wash. During third wash incubate the plate for 10 minutes before aspiration.

(xi) Add 50 μl horse radish peroxidase (HRP) conjugate antihuman IgG in dilution of 1:100 made in 1% BSA – TBS.

(xii) Shake the plate for 1 minute.

(xii) Incubate at room temperature for 30 minutes.

(xiv) Aspirate off the conjugate and repeat washing procedure as in step (x).

(xv) Add 50 μl of activated substrate solution to each well.

(xvi) Incubate micro titre plate for 30 minute at room temperature.

(xvii) Aspirate off the substrate.

(xviii) Wash the plate three times in TBS.

(xix) Allow the plates to dry.

Interpretation

Serum dilutions causing the development of well defined blue purple dots on antigen discs are considered positive.

Advantages

The advantages of this test over conventional ELISA test are that it is less time consuming, inexpensive and visually readable. This test is as sensitive and specific as conventional ELISA test.

11.5 Other indirect evidences

Changes in blood picture

Leucocyte count reveals leucopaenia (neutropaenia) with marked diminution of neutrophil granulocytes accompanied with a relative increase of lymphocytes and monocytes. Eosinophil granulocytes are absent, During the course of the disease there is a progressive diminution of leucocyte count falling to 1000/cmm of blood or even below that.

Erythrocytes also decrease in number. The proportion of leucocytes to erythrocytes is greatly altered and may be about 1:2000 to 1:1000 (Normal 1:750).

Non-specific serological tests

These tests depend on rise in gamma-globulin levels and become positive when disease is of three months duration. Different tests employed are :

Aldehyde test

(i) Take 1 to 2 ml of serum in a small glass test tube

(ii) Add a drop or two of 40% formalin

Jellification or milky white opacity like the white of a hard boiled egg within 2 to 20 minutes indicates a positive test.

Antimony test

(i) Take 1-2 ml patients's serum in a small glass test tube

(ii) Add few drops of 4% urea stibamine solution

Formation of profuse flocculant precipitate indicates positive test. This test is less reliable than aldehyde test.

However, a major disadvantage with these tests is their positivity in may other diseases where albumin : globulin ratio is reversed and are positive only when the disease is of 3 months duration.

11.6 Recent molecular techniques

The introduction of DNA probes and the advent of the polymerase chain reaction (PCR) have made it possible to detect and identify small numbers of parasites in tissues isolated from infected humans. DNA detection procedures could also be used to detect parasites in reservoir hosts, in other animals, and in infected sandflies.

PCR is being used for detection of amastigotes in tissue biopsy material from humans and animals.

DNA hybridization technique has also been used for detection of parasite DNA in infected tissues.

The above mentioned methods are available only at few centres mainly as research tools.

11.7 References

1. 'Leishmaniasis' in the Biology of Trypanosomes and Leishmaniasis parasites of man and domestic animals. Eds : Molyneux, O.H. and Ashford, R.W. 1983.

2. Sehgal Saraljit, Mittal Veena and Bhatia Rajesh; Leishmaniasis .: Manual on Laboratory techniques 1989.

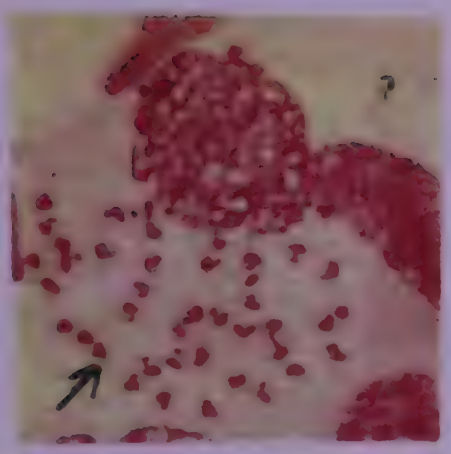


Fig. 11.1

LEISHMANIA DONOVANI
BONE MARROW
GIEMSA STAINING X 1200

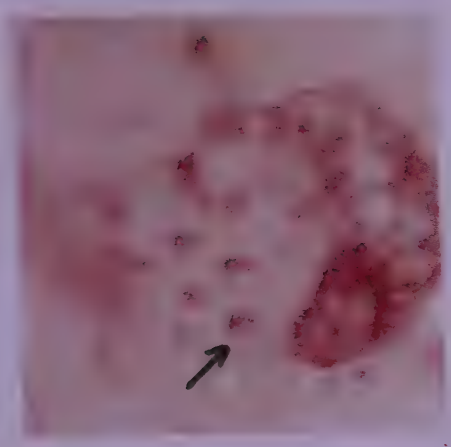


Fig. 11.2

LEISHMANIA TROPICA
SKIN BIOPSY
GIEMSA STAINING X 1200

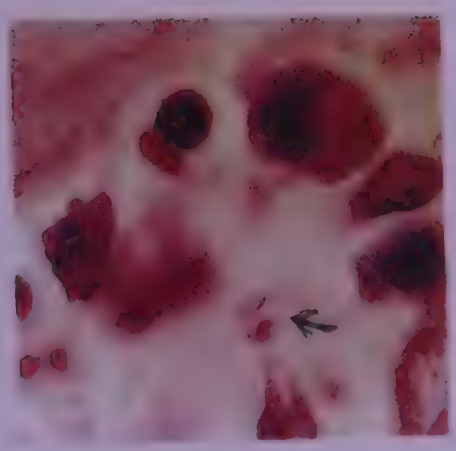


Fig. 11.3

LEISHMANI DONOVANI
TISSUE
GIEMSA STAINING X 1200

SECTION –III : FUNGAL INFECTIONS

Chapter –12

FUNGAL INFECTIONS IN HIV/AIDS

12.1 Introduction

The Acquired Immune Deficiency Syndrome (AIDS) due to the retrovirus HIV is characterised by the gradual loss of immune system functions. The hallmark of this process is a marked depression of cellular immunity. This often leads to several opportunistic infections including fungal infections. Studies on AIDS in the USA and Africa show that at least 50 to 90 percent of all patients contract a fungal infection at some time during the course of the illness and 10 to 20 percent die as a direct consequence of these infections[1]. The role of fungal infections became more important since Pneumocystis carinii, which previously thought to be a protozoan, has been classified as a fungus on the basis of genetic studies [2].

The range of mycoses seen in AIDS patients is very wide. Almost all of the well recognised fungal pathogens have been described in AIDS from different parts of the world, in addition to a number of various rare types. In many instances they differ either in extent or clinical presentation from the long established patterns of infections.

The most common and some of the uncommon mycoses seen in patients of AIDS are listed below:

Mycoses in AIDS Patients

Mycoses	Organisms	Main tissues involved
Common		
Candidiasis	Candida albicans	Oral mucosa, oesophageal mucosa
Cryptococcosis	Cryptococcus neoformans	Brain, meninges, lung
Histoplasmosis	Histoplasma capsulatum	Recticulo–endothelial system
Coccidioido mycoses	Coccidioides immitis	Lungs
Uncommon		
Aspergillosis	Aspergillus spp.	Lungs
Blastomycosis	Blastomyces dermatitidis	Lungs
Penicilliosis	Penicillium marneffei	Reticulo–endothelial system
Sporotrichosis	Sporothrix schenckii	Brain, skin
Dermatophytosis	Trichophyton spp. Epidermophyton floccosum	Nail, skin
Pneumocystis infections	Pneumocystis carinii	Lungs, eyes and other organs

While candidiasis, cryptococcosis, histoplasmosis and coccidioidomycosis are the major mycoses in patients of AIDS, the rest are uncommon except dermatophytosis. Coccidioidomycosis is a major mycosis in AIDS patients, but it is geographically limited to endemic areas (South Western

States of United States and elsewhere in the Western hemisphere). Although dermatophytosis is common in AIDS, its importance is doubtful. Candidiasis and Cryptococcosis are the major mycoses seen in AIDS patients in India.

12.2 Candidiasis

Candidiasis is an acute or chronic, superficial or disseminated fungal infection caused by species of Candida. The disease manifests in a variety of forms. Since the pathogenic species of candida forms the commensal flora of most individuals, the diagnosis of candidiasis should be carefully established. Up to 90% of HIV-positive individuals develop candidiasis at some time during the illness .[3] Candida infections in AIDS are usually limited to superficial candidiasis of varying degrees of severity in the oral cavity, throat and oesophagus. Recurrent often chronic vulvo vaginal candidiasis is well described in female patients with AIDS. But, in general, whether male or female, oral thrush is the most common form and appears when the CD4 lymphocyte count is about 200 mm^{-3}. The development of this condition is often the initial clinical manifestation in asymptomatic individuals and is one of several clinical signs that have been associated with an increased likelihood of progression to AIDS. Pulmonary and central nervous system candida infections have also been reported in a small number of AIDS patients.

Although C. albicans is the most important cause (superficial form) of candidiasis in patients of AIDS, many other members of the genus have also been recognised as pathogens.

Laboratory diagnosis

Source of material (specimen)

Material for examination vary considerably depending upon the type of clinical symptoms and may include mucous patches from mouth, vagina and arms, skin or nail scrapings, sputum, blood and cerebrospinal fluid (CSF).

Direct microscopy

A thin film of mucous material or sputum is made by pressing the material between two glass slides and stained with PAS or by Gram's method and examined for pseudomyceluim and budding cells (blastospores). Skin and nail scrapings are mounted in a 10-20% KOH sloution on a slide, covered with glass coverslip and heated gently before examination.

Culture

Material is cultured on Sabouraud dextrose agar with chloramphenicol at 37°C. Blood culture should be performed in all cases of suspected deep candidiasis. Identification of the species is based upon morphological features of the organism and also by assimilation and fermentation tests. Quick identification of C. albicans can be easily made through the germ-tube test (Fig. 12.1). For species identification the specimen can be sent to the Reference Centre.

Histopathology

Biopsy material showing tissue invasion by Candida is diagnostic of invasive candidiasis. Both yeast and mycelial forms are usually present.

Serology

Tests for the detection of antibodies against Candida are least helpful in severely immuno-suppressed patients including patients of AIDS. Consistently rising titres are suggestive of deep infection. Methods for the detection of circulating C. albicans antigens may be more useful. Kits are available commercially.

12.3 Cryptococcosis

Cryptococcosis is an acute, subacute or chronic pulmonary, systemic or meningeal mycosis. The disease could also rarely get disseminated to other organs, but involvement of central nervous system with meningitis is the most familiar form of the disease. The prevalence of crypotococcosis in patients of AIDS varies from country to country : 3–6% in Europe, 6–10% in the United States and in up to 30% in parts of Africa [4]. In AIDS, cryptococcosis is often insidious in onset, with few meningeal symptoms or signs. Often the sole presenting symptom is mild headache. Less than 20% are somnolent, confused or obtunded. In some AIDS patients, however, the neurological signs are rare.

Cryptococcus neoformans, an encapsulated yeast is the causal agent of Cryptococcosis.

Laboratory diagnosis

Source of material (specimen)

Sputum, CSF, urine, blood, pus from skin lesion and autopsy materials. All materials collected should be in sterile containers for laboratory examination.

Direct microscopy

A loopful of CSF, pus, sputum etc., is placed in a drop of India ink and covered with a cover slip and examined. Cells with capsules which may be twice as wide as the diameter of the cell are seen (Fig. 12.2).

Histopathology

In tissue cryptocci are present as a round yeast cell of 5–15 μm in diameter. The sections are stained with methenamine silver stain.

Culture

The organism could be isolated on Sabouraud dextrose agar containing chloramphenicol. Niger seed agar (Quizotia abyssinica seed) with creatinine should be used as a selective medium for the isolation of C.neoformans. In this medium C. neoformans will produce a brown colour.

Serology

The most effective test is the latex test for capsular antigen in CSF or blood. The antigen test is positive in over 90% of patients with untreated meningeal infection. Latex kits are available commercially.

12.4 Histoplasmosis

Histoplasmosis is a disease primarily involving the reticuloendothelial system. Infection may be localised or generalised. It may occur as primary histoplasmosis of the respiratory system or as progressive histoplasmosis with dissemination. Histoplasmosis is a serious problem in AIDS patients and in the USA it is reported in as high as 6% patients(disseminated histoplasmosis is the usual form). Common presenting symptoms are fever, lymphadenopathy splenomegaly and cough [5]. Histoplasma capsulatum, a dimorphic fungus, is the cause of histoplasmosis.

Laboratory diagnosis

Source of material (specimen)

Blood, bone–narrow, sputum, CSF or biopsy material. All materials should be collected in sterile containers for laboratory examination.

Direct microscopy

Detection of the organism in sputum by direct microscopy is difficult and often negative by KOH procedure. The organism can be seen in stained preparations (either Wright or Giemsa method Fig. 12.3 and 12.4). The organism is seen within macrophages and is 2–4 μm in diameter. It is usually ovoid with bud at the smaller end. Organisms tend to be much more abundant in peripheral blood smears and bronchial washings from AIDS patients.

Culture

Histoplasma capsulatum can easily be cultured from blood, bone-narrow, sputum, CSF, etc. Infected material is inoculated without delay on several media. Brain heart infusion agar with 6% blood without antibiotics at 37^{0}C, brain heart infusion agar with cycloheximide and chloramphenicol at 25^{0}C, should give suitable results. The cultures should be retained for six weeks before being considered as negative. The organism could be identified by demonstration of the characteristic tuberculate macroconidia and conversion of the mycelial to yeast phase at 37^{0}C.

Serology

Detection of circulating Histoplasma antigen is found to be a sensitive method for diagnosis of disseminated Histoplasmosis in AIDS. Antigen levels fall in patients receiving antifungal treatment and rise in those who have relapsed. Kits are available commercially.

12.5 Aspergillosis

Aspergillosis is a name given to a number of states such as allergic Aspergillosis, Aspergilloma, invasive and disseminated Aspergillosis. Although invasive/disseminated Aspergillosis has been reported in AIDS patients, it is much less common than Candidiasis or Cryptococcosis. The infection may result in necrotizing pneumonitis or spread widely and invade virtually any organ. Aspergillus species (mostly A. fumigatus) have been isolated from a large number of patients with HIV diseases[6], or identified at post mortem examination of patients with AIDS. In most cases Aspergillus infections co-exist with an HIV related malignancy or infection and is often undiagnosed until autopsy. The commonest clinical features include fever, cough, dyspnoea, chest pain and haemoptysis.

Laboratory diagnosis

Source of material (specimen)

Includes sputum, blood and skin scrapings.

Direct microscopy

A little of sputum and other samples are placed on to a clean slide in a drop of 10% KOH and examined for septate hyphae. The detection of non-pigmented septate filaments of 3–4 ìm which show repeated dichotomous branching is characteristic of Aspergillus infection.

Fig. 12.5 Shows the sketch of basic fungal morphology and Fig. 12.6 shows the sketch of Aspergillus spp.

Histopathology

The hyphal units of Aspergilli are stained reasonably well with Haematoxylin and Eosin. If the Aspergillus is growing in a cavity within an air-space (fungus wall), conidiophores and conidia are frequently observed.

Culture

Pathogenic species of Aspergilli can readily be isolated from pathological specimen on Sabouraud dextrose agar with chloramphenicol. The species of Aspergilli are identified on the basis of their colony colour and morphologic features.

12.6 Blastomycosis

Blastomycosis is a chronic granulomatous and suppurative disease having a primary pulmonary stage followed often by dissemination to other body sites such as skin and bone. AIDS patients have developed fulminant blastomycosis with wide spread dissemination following endogenous reactivation of previous infection. It is much less common than histoplasmosis.

Blastomyces dermatitidis which is also a dimorphic fungus, is the aetologic agent of blastomycosis.

Laboratory diagnosis

Source of material (specimen)

Skin scrapings, biopsy tissues, sputum, pus from the skin lesions and blood.

Direct microscopy

Specimens mounted in KOH are usually adequate to see the characteristic large round cells with thick refractile walls and broad-based single bud under the microscope.

Culture

The materials should be inoculated on the Sabouraud dextrose agar with chloramphenicol at room temparature and brain heart infusion agar at 37°C. The organims usually grow at room temparature as a mould, and as yeast at 37°C on brain heart infusion agar. The fungus can be identified by its size of the budding, yeast cells and ability to convert mycelial phase to yeast phase.

Serology

Serological tests are of limited usefulness in the diagnosis of Blastomycosis.

12.7 Penicilliosis and Sporotrichosis

Two other unusual fungal infections which are characterised by their presentation as disseminated disease in AIDS are Penicilliosis caused by Penicillium marneffei and Sporotrichosis due to Sporothrix schenckii. Most of the Penicillium marneffei infections in AIDS are reported from south east asia or those who visited the rural areas of south east asia[7]. Main clinical presentations of penicilliosis include fever, weight loss, cough, lymphadenopathy, hepatomegaly, leucocytosis and anaemia. Unfortunately, these symptoms are not specific because patients with AIDS usually present similar symptoms due to the HIV infection. Laboratory diagnosis of P. marneffei were made by direct microscopy and culture isolations. Source of clinical materials for the laboratory diagnosis include bone-narrow aspirate, blood and skin biopsy specimens. In microscopic observations of the Wright's stained specimen of bone-marrow, yeast cells of P. marneffei could be observed. Since the fungus is dimorphic it is identified on the basis of conversion of the the mycelial phase to yeast phase.

Disseminated Sporotrichosis caused by another dimorphic fungus Sporothrix schenckii, has been seldom reported in patients of AIDS. Clinical presentation of the illness usually includes weight loss, low-grade fever and development of wide spread erythematous cutaneous papules or nodules

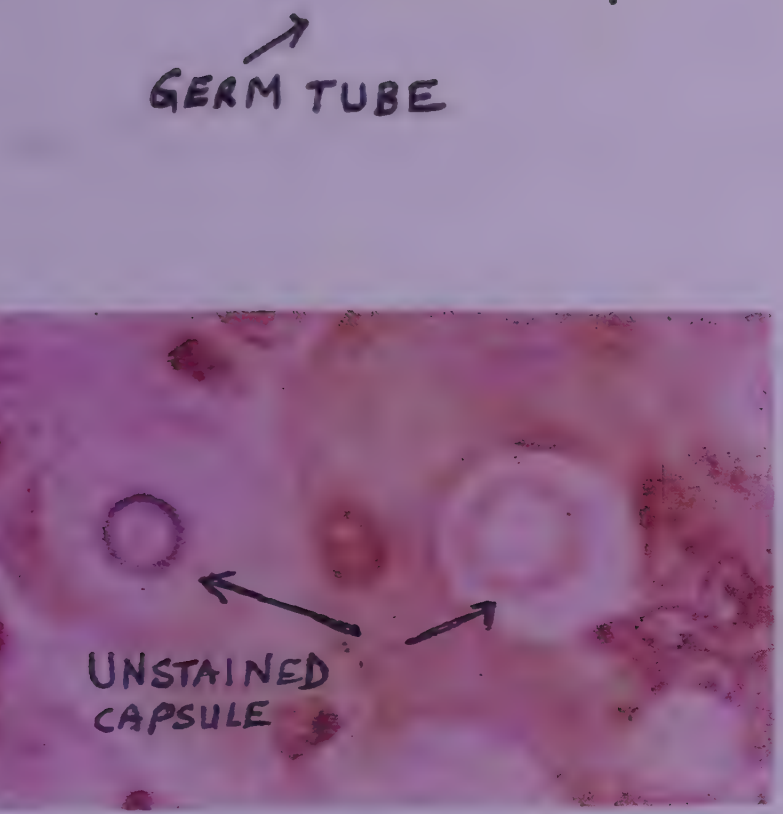


Fig. 12.1

CANDIDA ALBICANS

GERM TUBE

Fig. 12.2

CRYPTOCOCCUS NEOFORMANS IN CSF INDIA INK STAINING

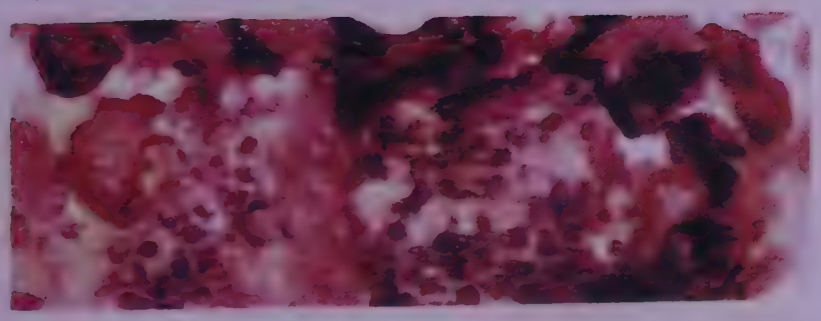


Fig. 12.3

HISTOPLASMA CAPSULATUM IN TISSUE X 1200 GIEMSA

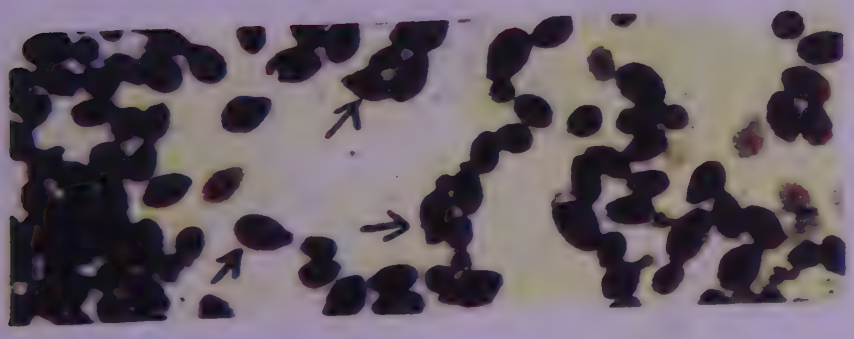
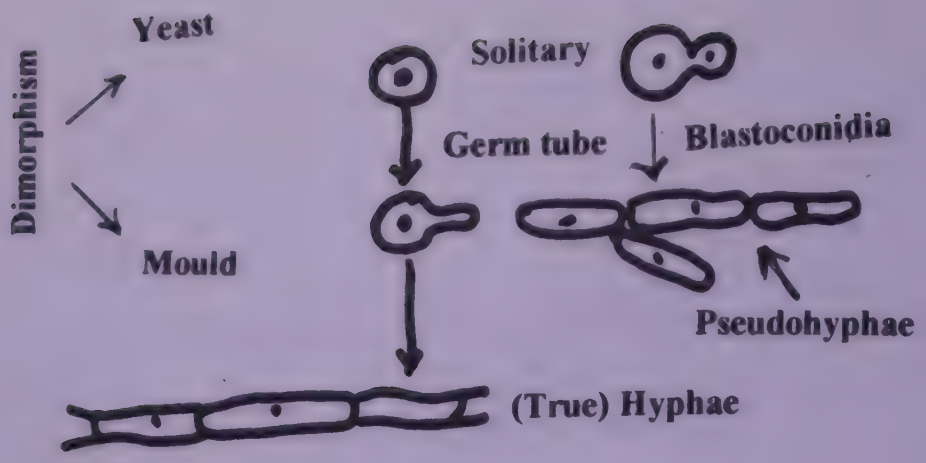


Fig. 12.4

HISTOPLASMA CAPSULATUM YEAST PHASE -SHOWING BUDDING X 1200 G.M.S. STAINING



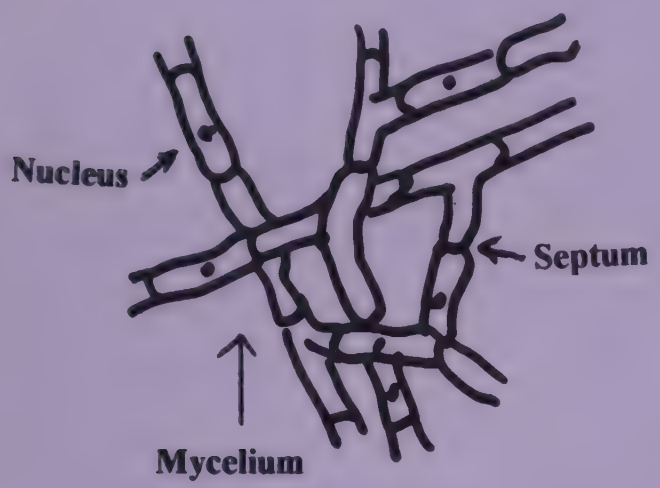


Fig. 12.5 BASIC FUNGAL MORPHOLOGY

Fig. 12.6 ASPERGILLUS SPP.

Fig. 12.7 PENICILLIUM SPP.

that ulcerate. For laboratory diagnosis skin biopsy specimens, besides being histologically examined, may be stained by periodic acid-schiff or methenamine silver stain. However, cultures are more often diagnostic. The fungus may also be cultured from blood and bronchial secretions. Here also, the fungus is identified on the basis of its diphasic character.

12.8 References :

1. Holmberg, K and Mayer, R.D. 1986. Fungal infectons in patients with AIDS and AIDS - related complex. Scand. J. Infect. Dis., 18 : 179-192.

2. Edman, J.C., Kovacs, J.A. Masur, H., Swati, D.V. and Elwood, H.J. 1988. Ribosomal RNA sequence shows Pneumocystis carinii to be a member of the fungi. Nature, 334 : 519.

3. Drouet, E and Dupont, B. 1989. Mycoses in AIDS patients : An overview (Abstract). Third symposium on topics in Mycology - Mycoses in AIDS patients. PP 20-23. The Pasteur Institute, Paris, France.

4. Moosa, M.Y.S. and Coovadia, Y.M. 1997. Cryptococcal Meningitis in Burban, South Africa: A comparison of clinical features, laboratory findings and outcome for Human Immunodeficdiency Virus (HIV) - Positive and HIV-Negative patients. Clinical Infectious Diseases, 24 : 131-134.

5. Graybill, J.R. 1988. Histoplasmosis and AIDS. The Journal of Infectious Diseases, 158 (3): 623-626.

6. Murray, J.F. and Mills, J. 1990. Pulmonary Infectious Complications of Human Immunodeficiency Virus Infections. Am Rev. Respiratory Dis., 141 : 1582-1598.

7. Suppartipinyo, K., Khamwan, C. Baosoung, V., Nelson, K.E. and Sirisanthana, T. 1994. Disseminated Penicillium marneffei infection in South East Asia. The lancet 344 : 110-113.

8. Drouhet, E and Dupont, B. 1990. Mycoses in AIDS Patients : An overview. In Mycoses in AIDS Patients. Eds. H.V. Bossche, D.W.R. Mackenizie et al. Plenum Press, New York, PP : 27-53

12.1 Appendix

10% KOH (Potassium Hydroxide)

Potassium hydroxide 10 g
Distilled water 100 ml

(i) Add potassium hydroxide crystals to distilled water slowly and with stirring
(ii) Mix by stirring until crystals are completely dissolved.

India ink

India ink is purchased in art supply stores. Pelican Drawing Ink, 17 Black, Gunther wagner, Germany, is recommended.

Periodic acid schiff stain (PAS)

Principle

Yeast and dermatophyte hyphae are effectively demonstrated in tissues with this stain.

Materials

(i) Four Coplin jars, each containing one of the following reagents :
  (a) Formalin–ethanol mixture
  (b) 1 % periodic acid
  (c) Schiff's reagent brought to room temperature
  (d) Light green working solution
(ii) One Coplin jar for water rinsing
(iii) Three wash bottles, each containing one of the following :
  (a) 70% alcohol
  (b) 95% alcohol
  (c) Absolute alcohol
(iv) Xylol in a dropper bottle
(v) Histoclad or permount mounting medium
(vi) Clean glass slide and cover slip
(vii) Control slide

Reagents for periodic acid–schiff stain

(i) Formal–ethanol mixture

40% formaldehyde	10 ml
Absolute alcohol	90 ml

(ii) Periodic acid, 1%

Periodic acid	1 g
Distilled water	100 ml

(iii) Schiff's reagent (commercially available)

Boiling distilled water	200 ml
Basic fuchsin	1 g

(a) Cool to 50°C and filter
(b) Add 20 ml 1N HCl (83 ml concentrated HCl/1,000 ml distilled water). Cool to 25°C
(c) Add sodium bisulfite, 1 g; store in screw-top bottle in dark for two days

(d) Add activated charcoal, 0.5 g; shake intermittently for one hour

(e) Filter

(f) Store in dark-coloured, tightly closed bottle, in refrigerator (5 years); pour into a Coplin jar for use. Solution may be reused until it turns pink, at which time it must be discarded.

(iv) Light green working solution

(v) 70% alcohol

(vi) 95% alcohol Use in plastic squirt bottles

(vii) Absolute alcohol

(viii) Xylol – use from dropper bottle

(ix) Mounting medium (Histoclad, Permount, or other mounting medium)

Method

(i) Prepare smear. Do not heat fix

(ii) Using forceps, place slide in Coplin jars of reagents and tap water as indicated

(a) Formalin-ethanol, 10 minutes. (This fixes the tissue)

(b) Running tap water, 5–10 minutes

Note : Sections that have been embedded in paraffin and rehydrated are immediately placed in periodic acid.

(c) 1 % periodic acid, 20 minutes

(d) Running tap water, 10–15 minutes

(e) Schiff's reagent, 20 minutes

(f) Running tap water, 10–15 minutes

(g) Light green working solution, 12 minutes

(iii) Wash slide with two rinses each from wash bottles of 70%, 95%, and absolute alcohol in sequence.

(iv) Wash slide with two rinses each of xylol from dropper bottle

(v) Place a drop of mounting medium on slide and cover with cover slip

(vi) Allow to harden one hour before examining slide

Note : Formalin-ethanol and periodic acid solutions may be reused as long as they are clear but for no longer than six months. Schiff's reagent is stored in the refrigerator and may be reused as long as it is clear but for no longer than six months. When it turns pink, it is no longer usable.

Interpretation

(i) Fungi stain a brilliant magenta.

(ii) Background is green.

(iii) Mucin also stains a brilliant magenta, making this stain unsuitable for staining undigested respiratory secretions.

Chapter –13

PNEUMOCYSTIS CARINII INFECTION AND AIDS

13.1 Introduction

The first indicators of AIDS epidemic way back in 1981 were clusters of cases of Pneumocystis carinii pneumonia (PCP) without the usual risk factors. During early stages of the epidemic 60% cases of AIDS had PCP as their presenting illness in the U.S.A. and 80% of all AIDS cases had PCP during their lifetimes. However, the prevalence of PCP has come down in the west with the institution of effective prophylactic regimes. Still PCP carries a mortality rate of 10% to 20%[1].

Pneumocystis carinii is a single celled eukaryotic organism which has been variously classified as protozoan, a fungus or an undifferentiated protist. However, molecular analysis has revealed that it is nearer to fungi than protozoa. P.carinii exists in cyst form and trophozoite form which may be small 1.5 to 2μ or large 3–5μ. The trophozoites may have small or big nuclei depending upon size. The life cycle of this organism is not exactly known, continuous human exposure to this organism is a rule as indicated by the appearance of antibody in more than 80% of humans during early childhood. It is an intra–alveolar extracellular parasite.

13.2 Magnitude of problem

PCP develops in HIV infected persons with severe immunosuppression. In a large cohort of asymptomatic HIV infected gay men who were not receiving preventive therapy 8.4% with CD4 counts <200 cells/μl developed PCP within 6 months and 18.4% within 1 year.

PCP has been reported from AIDS patients in India. One reason of low prevalence of PCP in India may be due to lack of expertise in diagnosis.

13.3 Clinical presentation

PCP has a subacute presentation in HIV infected persons. The most common symptoms include cough, chest tightness and exertional dyspnoea. The cough is non productive. A sensation of tightness usually accompanies the cough, night sweats may occur. Extrapulmonary P. carnii infections are rare and its symptoms are nonspecific or refer to the organ involved.

13.4 Diagnostic procedure

The diagnosis of PCP is by demonstration of the organism in pulmonary secretions.

13.4.1 Collection of specimen

Collection of correct specimen is very important for accurate diagnosis. As PCP patients do not have productive cough so, special procedures are required to obtain the correct specimen. Three categories of specimens are collected :

(i) Induced expectorated sputum or tracheobronchial suction (30% to 90% sensitivity).

(ii) Broncho–alveolar lavage (BAL) to obtain alveolar specimen (90% to 99% sensitivity).

(iii) Pulmonary biopsy specimens obtained via bronchoscopy (95% to 100% sensitivity).

Alveolar contents can be induced in expectorated sputum in patients by using an inspired hypertonic saline mist.

- The patient is asked to gargle with water to remove saliva, oral debris and contaminating organisms.
- The patient is made to breathe a mist of hypertonic saline (3%) generated by an ultrasonic nebulizer for 5–15 min.
- Patient is asked to cough and all material (2–3 ml) is collected and diluted with an equal volume of sterile water (distilled water).

In case the nebulizer is not available patient is asked to breathe deeply in vapour mixture created by mixture of 15% Sodium chloride solution and 10% glycerine for 5–15 minutes. All expectorated material is collected after deep coughing.

- The specimen is liquefied with dithiothreitol or used as such for preparing smears and doing staining.

BAL

The sensitivity of BAL specimen for detection of P.carinii is 90%–99%. BAL should be performed in two areas of lungs including the upper lobes. After the bronchoscope is placed and wedged, normal saline is instilled in 50 ml aliquots and immediately withdrawn with suction or syringe. The recovered specimen is centrifuged and stained for P. carinii[2].

Lung biopsy

A high diagnostic yield for PCP is provided by transbronchial biopsy. The proceudre needs expertise. It should only be done in patients in whom bronchoscopy is being done and who are on aerosolized pentamidine prophylaxis. This procedure is rarely indicated, though the sensitivity for diagnosis of PCP is high after this technique.

13.4.2 Methods

The various staining techniques undertaken for demonstrating P. carinii include :

- Wright i.e Giemsa staining
- Immunofluorescence staining(IF)
- Modified toluidine blue O staining
- Silver impregnation staining : Gomori's methanamine silver staining.

Giemsa stain

The smear is prepared as described below for IF staining and is stained with Giemsa stain as described in chapter 10. The smear is examined under oil immersion lens for the presence of cysts and trophozoites of P. carinii which are 5–8 μm thick walled, may contain upto 8 intracystic bodies (1–2 μm) termed sporozoites. Trophozoites or thin walled pneumocystis (1–4 μm) are free living, pleomorphic and contain small nucleus.

Direct immunofluorescence staining for detection of Penumocystis carinii

Requirements

- Fluorescence microscope with filter system for FITC and Evans blue
- Glass coverslips (24 X 60 mm)

- Automatic pipettes
- Petridish with moistened filter paper for moist inculation
- Staining cuvettes
- Vortex mixer
- Screwcapped centrifuge tubes
- Dithiothreitol or sputolysin
- Commercially available kit with following reagents :
  - FITC conjugated mouse anti-pneumocystis monoclonal antibodies
  - Buffered glycerine mounting medium
  - Phosphate buffered saline

Procedure

- Mix 2 ml of the induced sputum with 4 ml of diluted dithiothreitol (0.3% in PBS or 0.9% NaCl) or sputolysin
- Incubate the mixture at 37^{0}C for 5 min
- Vortex for 30 seconds
- 6 ml PBS is added and vortexed again for 30 seconds
- Centrifuge at 2500xg for 5 min
- Discard the supernatant
- Vortex the residual sediment for 10 seconds
- Transfer one drop (50 μl) to a clean glass slide and spread gently, to make a smear
- Air dry the smear
- Fix smear by covering with cold acetone (-20^{0}C) for 5-10 min
- The fixed smear can be stored for one month at -20^{0}C

The specimen in the form of acetone fixed smear is ready to be treated with FITC-labelled anti-pneumocystis MAB.

If the specimen is broncho-alveolar lavage or bronchial washings, follwing procedure is to be followed :

- Centrifuge 5 ml of the lavage or bronchial washings in screwcapped tubes at 3000xg for 10 min
- Discard supernatant
- Resuspend the sediment in 0.5 to 1.0 ml of sterile 0.05 M Tris-HCl buffer (PH 7.5) and vortex for 30 seconds
- Smear is made from this suspension and treated as above

Immunofluorescent staining

- Bring all the kit reagents to room temperature
- Cover the smear prepared from the specimen as above with 30 μl of anti-pneumocystis MAB reagent

- Place the slide in a humid chamber (petridish containng moist cotton) and incubate for 30 min at room temperature
- Immerse the slide in PBS for 5 min
- Air dry the smear
- Add 3 drops of mounting fluid over the dried smear and cover with a cover slip
- Examine the slide with fluorescence microscope under 40x or 100x objectives

Results and interpretation

The pneumocystis organisms are visible either as single or aggregates of extra cellular thick walled cysts with bright apple green fluorescence. The cyst aggregate may or may not be embedded in a brightly stained extra cellular matrix. Individual cysts may show a comma shaped or parentheses like structure. Cysts usually show peripheral green fluorescence.

Other developmental stages like mature sporozoites/trophozoites and precysts may also be seen.

Gomori methenamine silver stain (GMS)[3,4]

Requirements

Prepare reagents as follows :

(i) Chromic acid (10% solution)

- Chromic acid = 100g
- Distilled deionized water = 1000ml

Weigh out 100 g chromic acid and dissolve in 1 litre distilled water. Solution usable for up to 1 year.

(ii) Methenamine (3% solution)

- Hexamethylenetetramine = 12g
- Distilled deionized water = 400ml

Weigh out 12 g hexamethylenetetramine and dissolve in 400 ml distilled water. Solution usable for up to 6 months.

(iii) Silver nitrate (5% solution)

- Silver nitrate = 5g
- Distilled deionized water = 100ml

Weigh out 5 g silver nitrate and dissolve in 100 ml distilled water. Solution is usable for up to 6 months.

(iv) Stock methenamine–silver nitrate

- Methenamine (3% solution) = 400ml
- Silver nitrate (5% solution) = 20ml

A white precipitate forms when reagents are added together, but immediately clears with mixing. Store at 4°C. The solution is usable for up to 1 month.

(v) Borax (5% solution)

- Borax (sodium borate) = 5g
- Distilled deionized water = 100ml

Dissolve sodium borate in distilled water. Solution is usable for up to 1 year.

(vi) Sodium bisulfite (1% solution)

-	Sodium bisulfite	=	5g
-	Distilled deionized water	=	500ml

Weigh out sodium bisulfite and add to distilled water. Solution usable for up to 1 year.

(vii) Gold chloride (0.2% solution)

-	Gold chloride (chloroauric acid; Sigma Chemical Co.)	=	0.5g
-	Distilled deionized water	=	250ml

Weigh out gold chloride and add to distilled water. Solution usable for up to 1 year.

(viii) Sodium thiosulfate (2% solution)

-	Sodium thiosulfate	=	10g
-	Distilled deionized water	=	500ml

Solution usable for up to 1 year

(ix) Stock light green solution

-	Light green SF–Yellowish	=	0.2g
-	Distilled deionized water	=	100ml
-	Glacial acetic acid	=	0.2ml

Solution usable for up to 1 year

(x) Working light green solution

-	Stock light green solution	=	10ml
-	Distilled deionized water	=	40ml

Solution usable for up to 1 month

(xi) Fill two Coplin jars each with absolute ethanol and 95% ethanol. These jars may be sealed with screwcaps and the reagents can be reused until they become cloudy.

(xii) Fill two Coplin jars with xylene. These jars may be sealed and the xylene can be reused.

Staining procedure

(i) Immediately before each staining procedure, prepare working methenamine–silver nitrate as follows:

-	Stock methenamine–silver nitrate	=	37.5ml
-	Distilled deionized water	=	37.5ml
-	Borax	=	3ml

This solution must be prepared fresh in a Coplin jar for each set of slides stained. It is not reusable, even for a second staining procedure performed immediately afterward.

(ii) Turn on water bath allowing 45–60 min. to reach 80°C

(iii) Prepare smears and air dry. Heat fix all smears at 70°C for 10 min on a heating block

(iv) Fix smears in absolute methanol for a minimum of 3 min

(v) Place two Coplin jars, one containing 5% chromic acid (approximately 50 ml) and the other containing working methenamine solution into 80°C water bath for 5 min

(vi) Place smear into heated 5% chromic acid. Incubate in water bath at 80°C for 2 min

(vii) Remove slides from chromic acid and place in Coplin jar with distilled water. Rinse slides in three changes of distilled water

(viii) Place slides in 1% sodium bisulfite for 30 seconds

(ix) Rinse in three changes of distilled water

(x) Place slides in heated methenamine working solution. Incubate in water bath at 80°C for 4 ½ min

(xi) Place slides in Copln jar with distilled water. Rinse in three changes of distilled water

(xii) Wipe the back of slides with a paper towel to remove excess methenamine

(xiii) Tone in 0.2% gold chloride for 30 seconds; background lightens in this step

(xiv) Rinse in three changes distilled water

(xv) Place slides in 2% sodium thiosulfate for 30 seconds to remove unreduced silver

(xvi) Rinse in three changes of distilled water

(xvii) Counterstain in light green working solution for 30 seconds

(xviii) Rinse in three changes of distilled water

(xix) Dehydrate and clear for 30 seconds intervals in two changes each of 95% ethanol, 100% ethanol, and xylene, respectively

(xx) Place coverslips on slides with Permount while the slides are still wet with xylene. Slides are ready to be read.

Interpretation

Background will be green and Pneumocystis cysts will appear gray to black. They are spherical, may be punched in and cup shaped, and may show black parentheses-like structures in the centre. The cysts tend to occur in groups, often surrounded by foamy looking, green exudative back ground material.

Modified toluidine blue O stain for <u>Pneumocystis</u> <u>carinii</u>

(i) Work within a fume hood to prepare sulfation reagent as follow :

- Place a Coplin jar into a tub of cool tap water (not < 10°C)
- Pour 45 ml glacial acetic acid into the Coplin jar
- With a glass pipette, slowly add 15 ml concentrated sulfuric acid, being careful to avoid splashing. Mix gently with the pipette
- Seal the Coplin jar with petroleum jelly or use a tightly capped screwcap

The solution can be stored at room temperature and must be prepared fresh each week.

(ii) Prepare toluidine blue O stain as follow :

- Toluidine blue O actual dye (adjust amount for dye content of stain powder)	=	0.16g
- Distilled water	=	60ml
- Concentrated hydrochloric acid	=	2ml
- Absolute ethanol	=	140ml

Dissolve the dye in the water. Working within a fume hood, slowly add the hydrochloric acid. Add the ethanol last. The solution is stable for 1 year at room temperature.

Staining procedure

(i) Prepare the specimen (usually bronchoalveolar lavage material) by centrifuging for 15 min at 1500x g. Spread the sediment thinly across several slides. If bronchial washing is received, choose mucoid flecks to stain. For particulary mucoid material, the sediment may be treated with sputolysin and recentrifuged before making slides. Touch preparations of tissues are also suitable for staining. Allow the slides to air dry.

(ii) Stain a known positive control with each patient specimen. Slides may be heat fixed gently before staining, although this is not necessary.

(iii) Place the slides in the sulfation reagent for 10 min. With a glass rod or pipette, mix the reagent thoroughly immediately after adding slides and again after 5 min

(iv) Place the slides into a glass slide holder (or another Coplin jar) and rinse for 5 min by gently running cold tap water over the slides. Drain excess water by touching the slide edges to paper towels.

(v) Place the slides in the toluidine blue O for 3 min. Drain excess stain onto paper towels.

(vi) Dip the slides into a Coplin jar filled with 95% ethanol for a few (approximately 10) seconds, until most of the blue dye has run off.

(vii) Further decolourize the slides in absolute ethanol for approximately 10 seconds

(viii) Decolourize slides completely by passing them through two changes of xylene for approximately 10 seconds.

(ix) Wipe the backs and sides of the slides to remove excess xylene and immediately coverslip the stained area with a large coverslip (20x50 mm) using permount. Slides can be examined immediately. Pneumocystis cysts appear as round, reddish blue structures, approximately 5 ìm in diameter, often appearing punched in or cup shaped, and usually are found in clusters.

13.5 References :

1. Frame, P.T Pneumocystis 1996. Pneumocystis carinii infection and AIDS. In : Management of the HIV infected patient. Ed. Suzanne Crowe, Jennifer Hoy and John Mills. Cambridge University Press, U.S.A. PP 298–317.

2. Baughman R.P., Dohn M.N., Shipley. R et. al 1993 Increased Pneumocystis carinii recovery from the upper lobes in pneumocystis pneumonia. The effect of aerosol pentamidine prophylaxis. Chest; 103 : 426–432.

3. Walzer, P.D. 1993. Pneumocystis carinii : recent advances in basic biology and their clinical application. AIDS. 7 : 1293–1305.

4. Sydney, M., and Baron, E.J. 1986. Bailey and Scott's Diagnostic Microbiology. The C.V. Mosby Company. St. Louis PP 251–255 and 805.

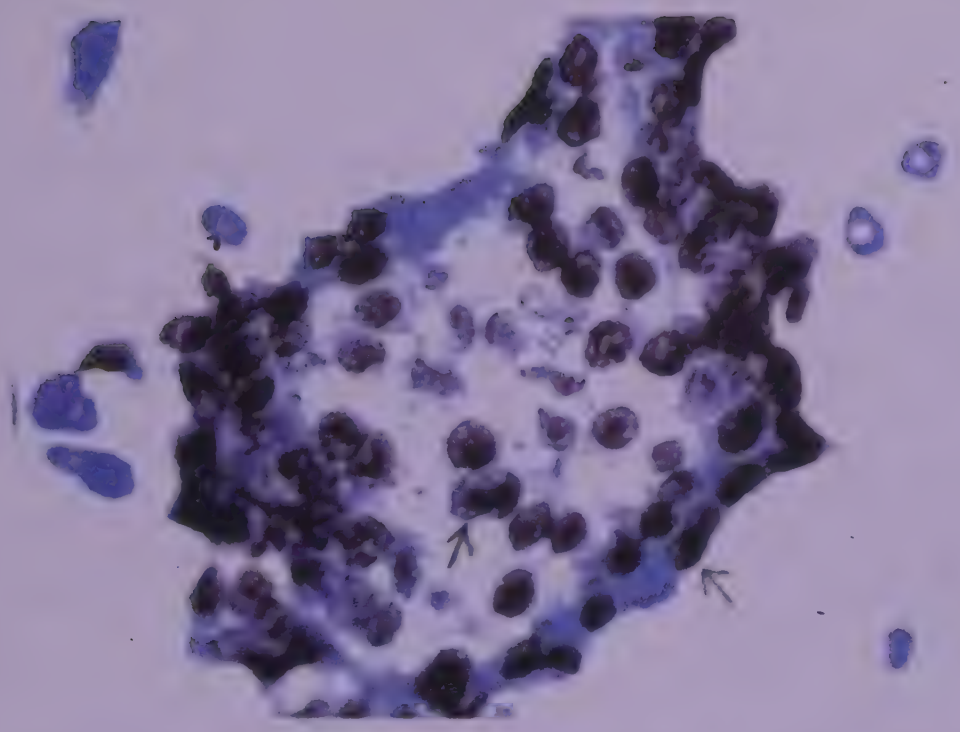


Fig. 13.1 PNEUMOCYSTIS CARINII (GMS STAIN) X 1200

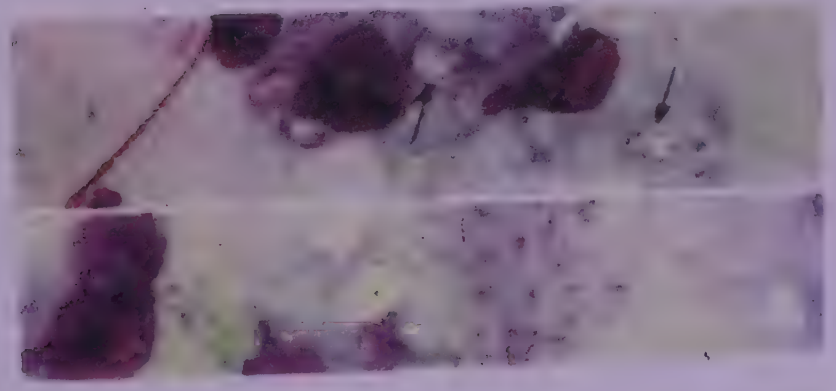


Fig. 13.2 PNEUMOCYSTIS. LUNG, GIEMSA, X1,200.

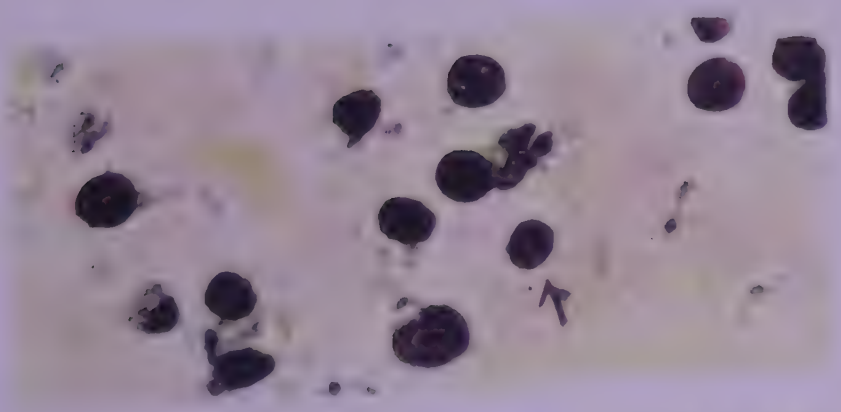


Fig. 13.3 PNEUMOCYSTIS. LUNG, GMS X 1200

SECTION –IV : BACTERIAL INFECTIONS

Chapter –14

TUBERCULOSIS AND MOTT IN HIV/AIDS

14.1 Introduction

Tuberculosis which had declined in the nineteenth and early twentieth century, has re-emerged during the last two decades of twentieth century on account of the advent of human immunodeficiency virus[1] all over the world.

14.2 Magnitude of the problem

The first report of tuberculosis occuring in patients with AIDS appeared in 1983 wnen an illness associated with severe immunosuppression was described in a group of Haitian patients in South Florida[2]. Of the 20 patients studied 10 had evidence of tubeculosis at the time their immunocompromised status was detected or earlier. In another description of AIDS in Haiti, 24% patients had tuberculosis usually preceding the diagnosis of AIDS[3].

Today tuberculosis is the most common HIV associated lung disease[4]. Tuberculosis, particularly Mycobacterium avium complex infections are frequently reported from the west in HIV/AIDS patients.

Indian scenario

Very little data is available in our country about patients dually infected with HIV and tuberculosis. However, various studies have reported a prevalence ranging between 14.6% to 67% in HIV infected individuals[5-6]. An analysis of 8000 odd cases of AIDS reported to NACO revealed tuberculosis to be a major opportunistic infection (63%).

14.3 Multidrug resistant tuberculosis (MDR-TB)

The re-emergence of multidrug resistant tuberculosis has compounded the problem of tuberculosis leading to a high rate of morbidity and mortality. Factors contributing to recent outbreaks and continuous spread of MDR tuberculosis include the emergence of AIDS in addition to inefficient infection control procedures and laboratory delays in identification and susceptibility testing[1]. The highest rates of MDR-TB are reported from Nepal (48%), Gujarat and New York City (30%)[7]. In India itself the prevalence of MDR-TB has increased from zero in 1980 to 10.7% in 1995[8]. However, no data is available on the prevalence of MDR-TB with HIV infection in India.

14.4 Nontuberculous mycobacteria in HIV infected patients

Nontuberculous mycobacteria (NTM) have often been identified as causing disease in HIV infected patients. However, the incidence of infection due to NTM other than M. avium intracellulare is low. Relatively non-virulent mycobacteria including M. Kansasii, M. simiae, M. genavense, M. haemophilum and M. scrofulaceum have also proved to be opportunistic pathogens in patients with AIDS[9].

M. avium intracellulare complex

Infection due to M. avium intracellulare (MAC) was relatively rare in the pre AIDS era. The most common manifestation of MAC infection was pulmonary. However, with the advent of AIDS, disseminated infection with MAC is recognized as the most common systemic bacterial infection in

patients of AIDS. Disease due to MAC is geographically widespread with reports of MAC in HIV infected patients from North and South America, Europe and Australia[10].

M. kansasii

M. kansasii is not as widespread as MAC. In the United States, it is the second most common NTM infection in patients of AIDS[11]. Early reports of M. kansasii in HIV infected individuals focussed on the dissemination and fatality due to M.kansasii. Pulmonary disease is more common than extrapulmonary disease in patients of HIV coinfected with M. kansasii[12].

14.5 Other nontuberculous mycobacteria

Very few studies are available on other NTM infection in patients of AIDS. M. haemophilum infections have been reported, mainly causing erythematous nodular skin lesions, subcutaneous abscesses, lymphadenitis and occasional joint involvement. M. genavense causes infection rarely, leading to bacteraemia in patients of AIDS. M. gordonae, one of the least pathogenic mycobacteria, can also lead to pulmonary infection. Scattered reports are available on the occurrence of M. scrofulaceum and M. celatum infections in patients of AIDS[12].

14.6 Clinical features

The clinical manifestations of tuberculosis in patients with HIV infection vary considerably depending on the severity of the immunosuppression[3-4]. The manifestations vary from typical pulmonary presentation in those with relatively well preserved immunity and CD4 counts > 200 cells/mm^3, to atypical disseminated disease in markedly immunosuppressed individuals.

Pulmonary disease : Bilateral lower lobe infiltration and pleural effusions are more frequent in patients of tuberculosis coinfected with HIV than others. Cavity formation and predilection for apical regions are less common.

Extrapulmonary diseases : As compared to only 10% of immunocompetent individuals about half of the HIV-TB cases have extrpulmonary manifestations. Lymphadenopathy is very common. Pericardial disease, meningitis and miliary disease may be the other manifestations.

14.7 Diagnosis

(i) Clinical

(ii) Radiological

(iii) Tuberculin skin test

(iv) Bacteriological methods

Tuberculin test needs to be interpreted with caution in patients with concurrent HIV infection. Though, a positive tuberculin test increases the likelihood of presence of tuberculosis, a negative test does not exclude the diagnosis.

14.7.1 Specimen collection and transport

The successful isolation and identification of the organism, as in all microbiological procedurs depends on the quality of the specimen obtained and appropriate processing of the samples. Specimens should be collected in sterile, leak proof, disposable and appropriately labeled containers.

Specimen

Pulmonary

- Laryngeal swab
- Spontaneously produced or induced sputum
- Transtracheal aspirate
- Bronchial aspirate
- Bronchial lavage

These are specimens of choice, specially for detecting NTM in patients of AIDS

- Gastric lavage : Limited to senile, non-ambulatory patients, children less than 3 years and other patients who may have swallowed sputum or are unable to produce sputum by aerosol induction. Three consecutive specimens are to be submitted within 3 days for processing.

Extrapulmonary

Blood

HIV infected patients can have disseminated mycobacterial infection, the most common species being M. avium complex. Blood is collected in broth such as radiometric BACTEC 13 A or the lysis centrifugation system.

Urine

Three consecutive morning specimens (entire volume) are submitted in sterile containers after proper precautions in case of urogenital tuberculosis.

Faecal specimens

Patients of AIDS at risk of developing disseminated MAC disease can be identified by acid fast staining and culture of faecal specimens.

Cerebrospinal fluid

At least 10 ml of CSF should be collected in case of tubercular meningitis.

14.7.2 Direct smear examination

Cheesy, necrotic and blood tinged material in the specimen is selected as it is more likely to produce positive results.

Staining methods

Ziehl-Neelsen stain

Materials required

Carbol fuchsin

Basic fuchsin	=	5g
Phenol	=	25g
95% ethanol	=	50ml
Distilled water	=	500ml

Dissolve the basic fuchsin in phenol by placing over a boiling water bath for 5 min., shaking the contents from time to time. Add alcohol after the solution has been made. Mix thoroughly. Add distilled water. Filter before use.

Acid alcohol

Hydrochloric acid (concentrated)	=	3ml
95% ethanol	=	97ml

Counterstain

Methylene blue	=	0.3g
Distilled water	=	100ml

Procedure

(i) Prepare the smear by spreading the material over an area 1x2 cm. Air dry and heat fix the smear.

(ii) Flood the slide with carbol fuchsin.

(iii) Heat the slide to steaming with a Bunsen burner and let stand for 5 min. If the smear dries, add more stain.

(iv) Wash the slide with water.

(v) Flood the slide with acid alcohol and allow to decolourize for two min.

(vi) Wash the smear with water.

(vii) Flood the smear with methylene blue and counterstain for 1 to 2 min.

(viii) Wash with water and air dry. DO NOT BLOT DRY.

(ix) Examine under oil immersion objective lens of the microscope.

Kinyoun stain

Materials required

Carbol fuchsin

Basic fuchsin	=	4g
Phenol	=	8g
95% ethanol	=	20ml
Distilled water	=	100ml

Prepare carbol fuchsin as described for Ziehl-Neelsen stain.

Acid Alcohol

Hydrochloric acid (concentrated)	=	3ml
95% ethanol	=	97ml

Counterstain

Methylene blue	=	0.3g
Distilled water	=	100ml

Procedure

(i) Prepare the smear as described above. Air dry and heat fix.

(ii) Flood the slide with Kinyoun carbol fuchsin for 5 min. No heating required.

(iii) Wash with water and decolourize with alcohol for 2 min.

(iv) Rinse with water and drain.

(v) Flood the smear with methylene blue and counterstrain for 1 to 2 min.

(vi) Wash with water and air dry.

(vii) Examine under oil immersion objective lens of the microscope (1000x).

Fluorochrome staining

Materials required

Soluton A

Auramine O	=	0.1g
95% ethanol	=	10ml

Solution B

Phenol	=	3g
Distilled water	=	87ml

Mix solutions A and B.

Decolourizing agent

Hydrochloric acid (concentrated)	=	0.5g
70% ethanol	=	100ml

Potassium permanganate

$KMnO_4$	=	0.5g
Distilled water	=	100ml

Procedure

(i) Prepare the smear as described earlier. Air dry and heat fix.

(ii) Flood the smear with auramine O solution and allow staining for 15 min. Do not heat.

(iii) Wash with distilled or deionized water as the chlorine in tap water may interfere with the fluorescence.

(iv) Decolourize with acid alcohol for 2 min.

(v) Wash and flood the smear with potassium permanganate and counterstain for two min. Do not counterstain for long as that may quench the fluorescence of acid fast bacilli.

(vi) Wash and air dry.

(vii) Observe by fluorescence microscopy (450x) (Fig. 14.2).

Examination of smears

To ensure that an area is covered only once, the smear should be searched in an orderly manner. To achieve this the microscopist should examine three parallel sweeps along the length of the smear or nine parallel sweeps along the width of the smear, searching 300 fields in all (Fig. 14.3).

Table –14.1 Acid fast smear reporting

No. of AFB seen in Ziehl–Neelsen stain (1000x magnification)	No. of AFB seen in Fluorochrome staining (450x magnification)	Report
0	0	No AFB seen
1–2/300 fields	1–2/70 fields	Doubtful. Request a fresh specimen.
1–9/100 fields	2–18/50 fields	1+
1–9/10 fields	4–36/10 fields	2+
1–9/ fields	4–36/ fields	3+
>9/ field	>36/ field	4+

(from Forbes BA, Salm DF and Weissfeld AS. 1998 Diagnostic Microbiology.)

14.7.3 Decontamination of specimens and culture

Before inoculation on to culture media, specimens such as sputum which are contaminated with bacteria other than mycobacteria must be treated by a method that kills other bacteria but not mycobacteria.

Petroff's method

Materials required

4% NaoH

NaoH	4g
Distilled water	100ml

Sterilize by autoclaving.

2 N HCl solution

Concentrated HCl	33ml
Distilled water	200ml

Phenol red indicator

Phenol red solution

(0.4% in 4% NaOH)	20ml
Concentrated HCl	85ml
Distilled water	895ml

Procedure

(i) Transfer 10ml sputum to a screwcap test tube.

(ii) Add equal volume of 4% NaOH solution and mix thoroughly by shaking.

(iii) Place the container in an incubator at 37^0 C for 30 min. Remove briefly for further mixing at intervals.

(iv) Centrifuge at 3000g for 15 min.

(v) Decant supernatant fluid.

(vi) Add a drop of phenol red solution to the sediment and neutralize with hydrochloric acid.

(vii) Mix sediment and inoculate on to the desired medium.

Culture

Culture media used for cultivation of mycobacteria :

Solid

Egg based medium

- Lowenstein Jensen medium
- Lowenstein Jensen medium with pyruvic acid
- Dorset's egg medium

Agar based medium

- Middlebrook 7H10 and Middlebrook 7H10 selective.
- Middlebrook 7H11 and Middlebrook 7H11 selective.

Liquid

- Middlebrook 7H9 broth
- BACTEC 12 B medium
- Septi-check AFB medium

Preparation of Lowenstein Jensen medium

Materials required

Mineral salt solution

Potassium dihydrogen phosphate	
KH_2PO_4 (anhydrous)	2.4g
Magnesium sulphate	0.24g
Magnesium citrate	0.6g
Asparagine	3.6g
Glycerol	12ml
Water	600 ml

Dissolve the ingredients with heating. Autoclave to sterilize.

Malachite green solution

2% malachite green in sterile water. Allow to dissolve by holding at 37^0C for 1-2 h.

Complete medium

Mineral salt solution	600ml
Malachite green solution	20ml
Egg fluid	1litre

Distribute in 28 ml McCartney bottles and screw on the cap tightly. Slant tubes and coagulate by inspissation at 85°C for 50 min.

Inoculation

Transter 2–3 drops of the processed specimen to the prepared medium and incubate at 37°C. All media should be inspected after 1 week to detect rapidly growing mycobacteria. There after all cultures should be incubated for 8 weeks with weekly examination for evidence of growth (Fig. 14.4).

Efforts have been made to develop rapid methods of culture. Welch et. al described a method using thinly poured Middlebrook 7H11 agar medium. Growth can be identified microscopically as early as 10 days. Radiometric culture techniques can shorten this time to 7 days. BACTEC 460 TB system contains culture media with ^{14}C labeled Palmitic acid. Mycobacteria if present metabolize the ^{14}C labeled substrates and release radioactively labeled $^{14}CO_2$ which is detected and quantified into a growth index according to the manufacturer's instructions.

14.7.4 Biochemical identification

14.8 Newer diagnostic techniques

14.8.1 DNA probes

In this technique, a nitrocellulose membrane filter is laden with the DNA extracted from the clinical specimens and a radiolabeled DNA probe complementary to a chosen suquence of target mycobacterium is added. After hybridization the radioactivity is measured by autoradiography. However, 10^4 bacilli/ml must be present for detection by this method[14]. Non isotopically labeled DNA probes using acridium ester as the label and specific for mycobacterial RNA sequences are commercially available (Gen probe). Presently nucleic acid probes are available against M. tuberculosis complex, M. avium intracellulare, M. kansasii and M. gordonae[15].

14.8.2 Polymerase chain reaction (PCR)

PCR is a recent sensitive and specific method for diagnosis of tuberculosis. the technique consists of repetitive cycles of denaturation of the native double stranded target DNA at 94° C, annealing of DNA primers to the complementary sequences at 50° C and extension of annealed primers by Taq polymerase at 72°C. Since the technique is expensive and requires trained personnel, it is used mainly by referral centres only.

14.8.3 Ligase chain reaction

Unlike PCR, ligase chain reaction amplifies the nucleic acid probe rather than the target sequence. The technique uses two pairs of probes that span the target sequence of interest. When the probe is annealed to the target sequence, a ligation reaction closes the space between the probes. On heating the joined probes are released as a single strand complementary to the target sequence and can be amplified and detected[15].

Table 14.2 Biochemical reaction of mycobacteria commonly encountered in patients of TB–HIV

Group complex	Species	Niacin	Growth on TCH (10μg/ml)	Nitrate reduction	Semiquan-titanitive catalase (>45mm)	68°C catalase	Tween hydrolysis (5 days)	Tellurite reduction	Tolerance to 5% NaCl	Iron uptake	Aryl sulphatase (3 days)	Growth on Mac Conkey agar	Urease	Pyrazin-amidase (4 days)
TB Complex	*M. tuberculosis*	+	+	+	–	–	–	±	—	—	—	—	+	+
	M. bovis	—	—	—	—	—	—	±	—	—	—	—	+	—
Photo-chromogens	*M. marinum*	±	+	—	—	—	+	±	—	—	±	–	+	+
	M. kansasii	–	+	+	+	+	+	±	–	–	–	–	+	–
	M. simiae	+	+	–	+	+	–	+	–	–	–	–	+	+
Scoto Chromogens	*M. scrofulaceum*	–	+	–	+	+	–	±	–	–	v	–	v	±
	M. gordonae	–	+	–	+	+	+	–	–	–	v	–	v	±
Nonphoto-chromogens	*M. avium complex*	–	+	–	–	±	–	+	–	–	–	±	–	+
	M. genavense	–	+	–	+	+	+			–	–		+	+
	M. haemophilum	–	+	–	–	–	–	–	–	–	–	–	–	+
	M. xenopi	–	+	–	–	+	–	±	–	–	±	–	–	v
Rapidly growing myco-bacteria	*M. fortuitum*	±	–	+	+	+	v	+	+	+	+	+	+	+
	M. chelonae	v	–	–	+	v	v	+	–	–	+	+	+	+

14.9 Indirect methods of detection

Serodiagnositic techniques

Enzyme linked immunosorbent assay (ELISA)

A number of scientists have detected antibodies to M. tuberculosis using crude antigens of the bacilli e.g. M. tuberculosis culture filtrate or BCG sonicate. some workers have also used the purified protein derivative as the antigen. The sensitivity of ELISA is still low in smear negative pulmonary tuberculosis (53% to 62%) and extrapulmonary tuberculosis (30% to 40%) with a specificity above 95%. Hence antibody detection ELISA is of limited use for detection of tuberculosis[15].

Radioimmunoassay (RIA)

RIA has a higher sensitivity than ELISA for both antigen and antibody detection. However, the technique is highly expensive with the added disadvantage of radioactivity and hence of limited use[15].

Demonstration of biological products

Adenosine deaminase detection

The enzyme adenosine deaminase (ADA) is ten times higher in lymphocytes than in RBCs. In addition the activity is more in T cells than in B cells. The activity of ADA is maximum in rapidly proliferating lymphocytes and rises whenever an antigen stimulates the cell mediated immunity[15].

Tuberculostearic acid

Tuberculostearic acid is found in the cell wall of mycobacterium and can be identified by gas chromatography or mass spectrophotometry. Although the method has a high sensitivity (>95%) and specificity (>99%), it has not been in routine use because it is a complex and costly technique.

Table -14.3 Comparision of various diagnostic techniques used for detection of mycobacteria

Test	Sensitivity	Specificity	Time required	Cost
AFB smear	Low	No *	Minutes	Inexpensive
Conventional culture	Moderate	Yes	Weeks	Inexpensive
Radiometric culture	Moderate	Yes	Days to weeks	Expensive
DNA probe	Moderate to high	Yes	Hours**	Expensive
RNA probe	Moderate to high	Yes	Hours**	Expensive
PCR+DNA probe	High	Yes	Hours	Expensive
ELISA	Moedrate	Hours	Less costly	

* Species identification is not recommended by smear examination, hence declared as of no specificity.
** Requires growing culture, hence the actual time required for detection increases. (Modified from Reichman LB and Hershfiled ES. Tuberculosis : A comprehensive International approach. Lung biology in Health and Research, Vol. 66 New York. Marcel Dekker, 1993).

14.10 Biosafety in the laboratory

Work related infections are a recognized hazard for personnel employed in laboratories where infectious disease agents are handled. The risk of tuberculosis infection is three to five times greater

for the microbiology laboratory worker than for other personnel in the same Institution. Handling TB–HIV cases requires even greater care.

An effort should be made to control the exposure of employees to pathogenic organisms for their safety, identifying the following tasks :

(i) Employee education and orientation

(ii) Appropriate disposal of hazardous waste

(iii) Universal precautions

(iv) Use of biological safety cabinets

(v) Personal protective equipement such as laboratory coats and gloves

(vi) Investigation of all accidents to prevent recurrences

Disposal of hazardous waste

All materials contaminated with potentially infectious agents should be decontaminated before disposal. These include specimens, patient cultures, stock cultures and disposable sharp instruments. **Infectious waste can be decontaminated in an autoclave or incinerator.**

Universal precautions

These include:

(i) Barrier protection

(ii) Hand washing

(iii) Safety techniques

(iv) Safe handling of sharp items

(v) Safe handling of specimens

(vi) Safe handling of spill of body fluids/blood

(vii) Safe techniques including mechanical pipetting

Biological safety cabinets

Processes dealing with M. tuberculosis are included in the biosafety level 3 procedures. Besides taking the previously described precautions, personnel working with M. tuberculosis should handle specimens and cultures in a class II biosafety cabinet.

14.11 References

1. Jacobs RF. Multiple durg resistant tuberculosis. Clin Infect Dis. 1994; 19 : 1–10.

2. Hopewell PC. Tuberculosis and infection with human immunodeficiency virus. In: Reichman LB and Hershfield ES (Eds), Tuberculosis : A Comprehensive International Approach. Lung Biology in Health and Research, Vol 66. New York : Marcel Dekker, 1993.

3. Pitchenik AE, Fischl MA, Dickinson GM, Becker DM, Fournier AM, O'Connell MT, Colton RM and Spira TJ. Opportunistic infections and Kaposi's sarcoma among Haitians : Evidence of a newly acquired immunodeficiency state. Ann Intern Med. 1983; 98 : 277–284.

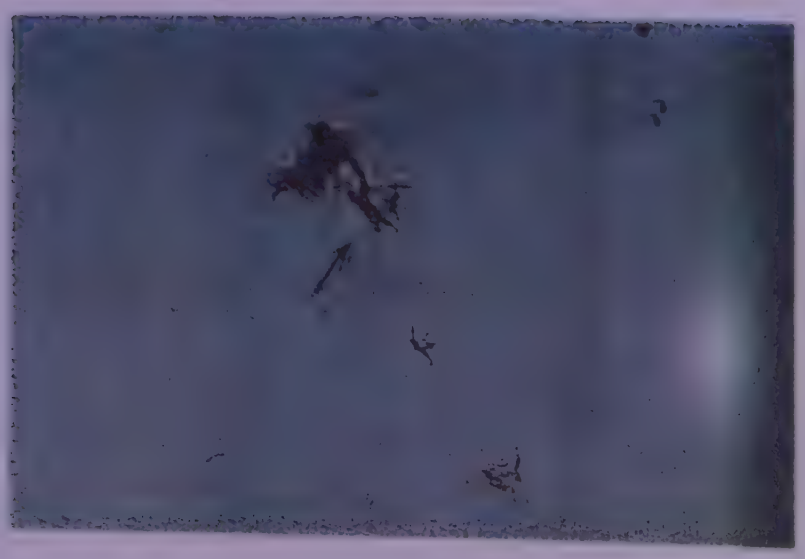


Fig. 14.1

Fig. 14.2

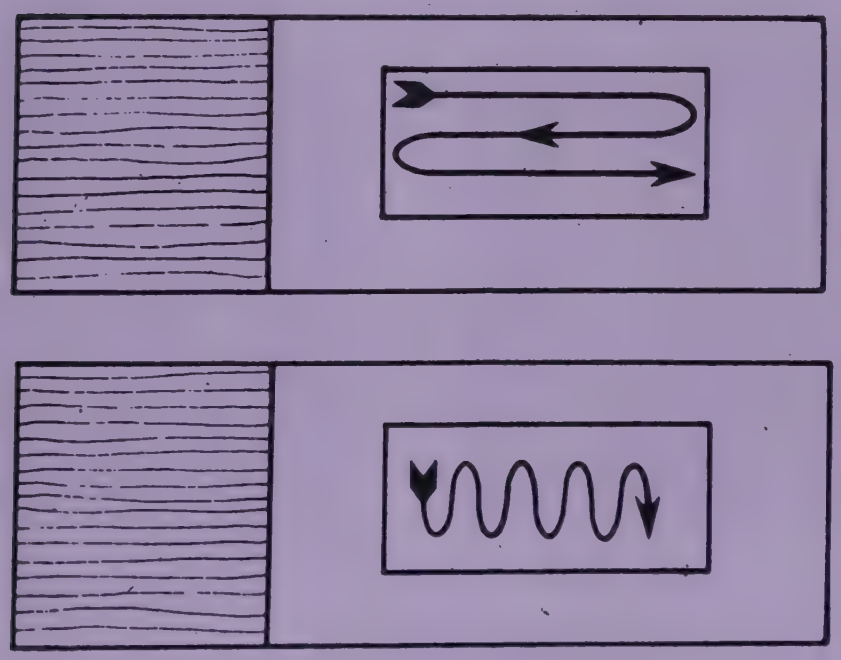
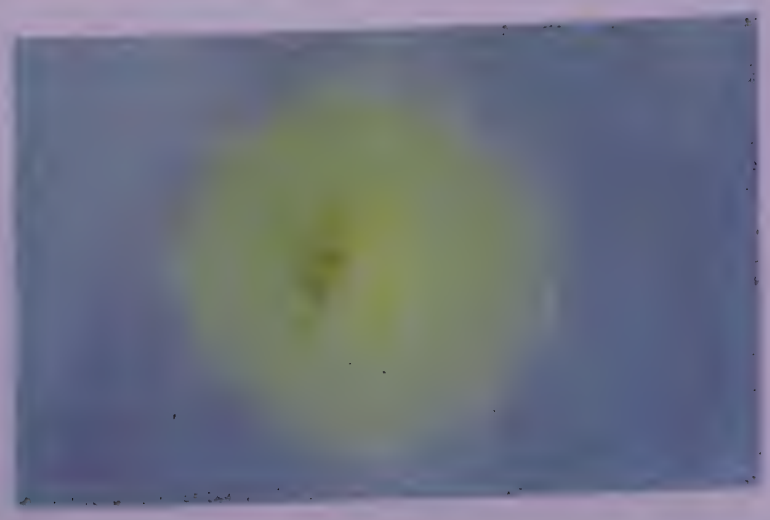


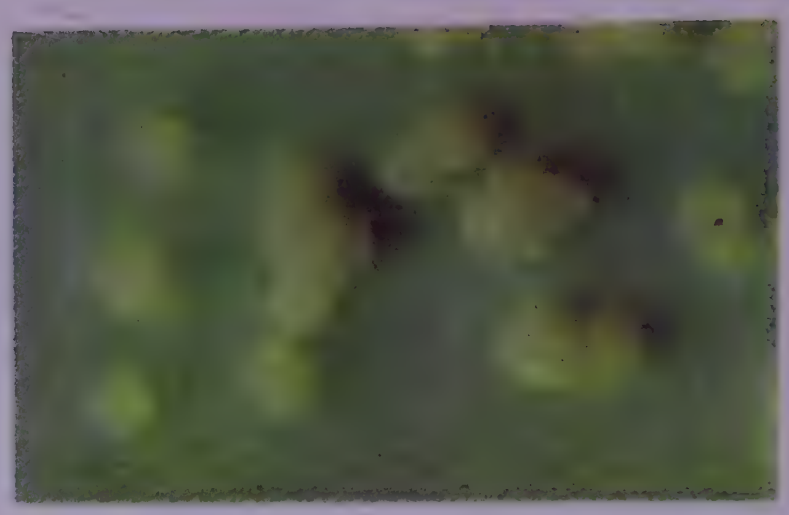
Fig. 14.3



M. TUBERCULOSIS - ROUGH, DRY BUFF COLOURED COLONIES ON LOWENSTEIN JENSEN MEDIUM

M. BOVIS - ROUGH, DRY COLONIES ON LOWENSTEIN JENSEN MEDIUM

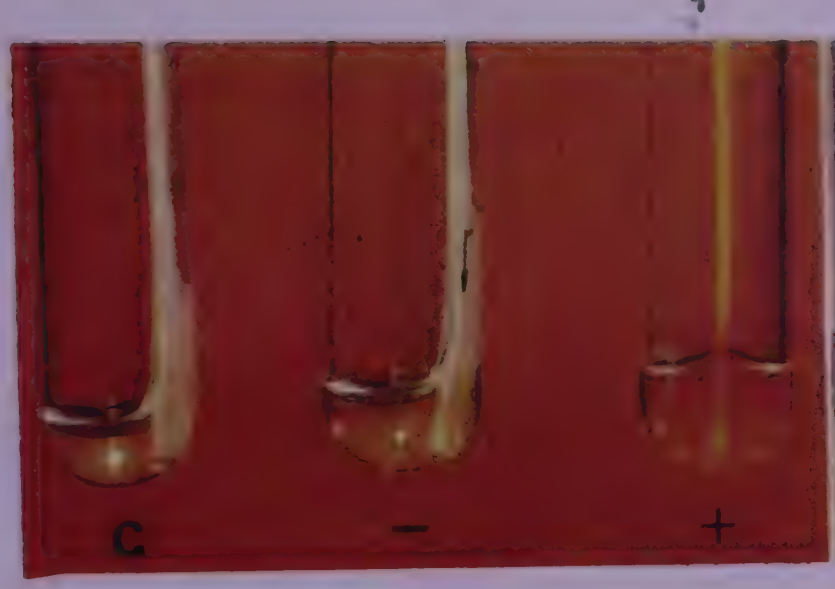
M. AVIUM COMPLEX - SMOOTH, DOMED COLONIES ON LOWENSTEIN JENSEN MEDIUM



M. KANSASII - ROUGH COLONIES ON LOWENSTEIN JENSEN MEDIUM

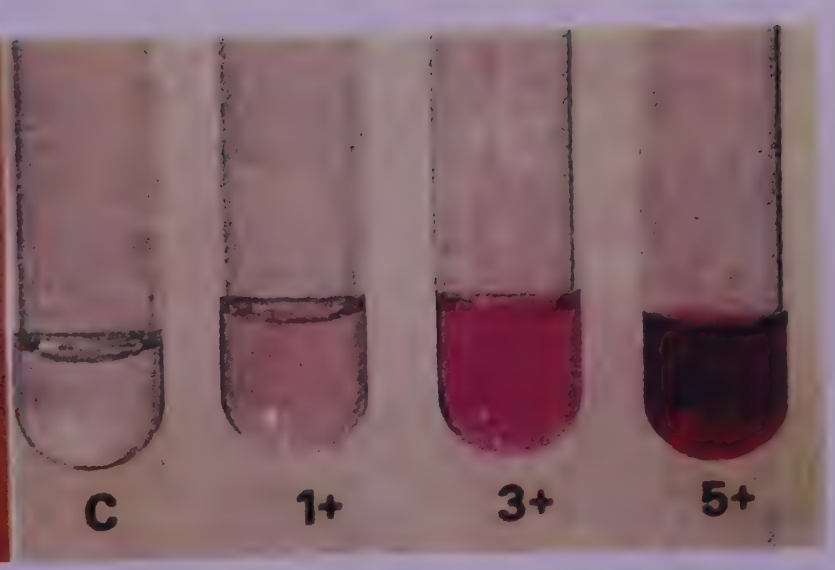
Fig. 14.4

BIOCHEMICAL REACTIONS



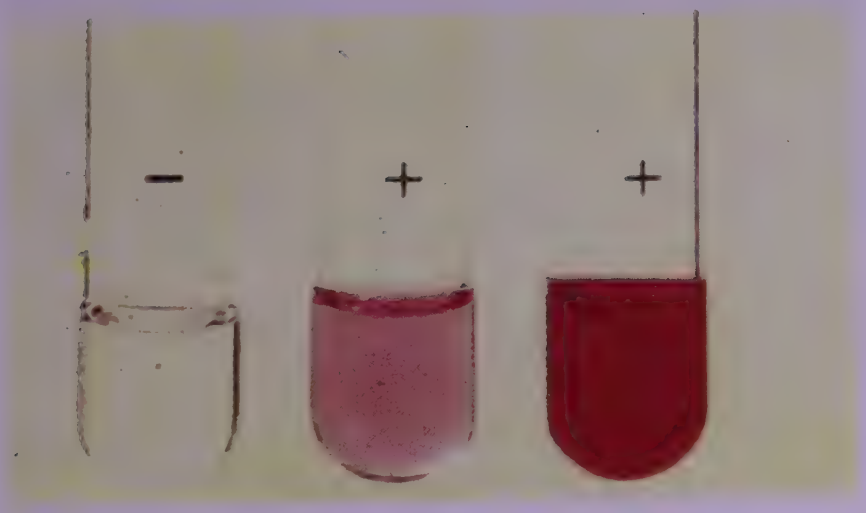
NIACIN PAPER STRIP TEST

A



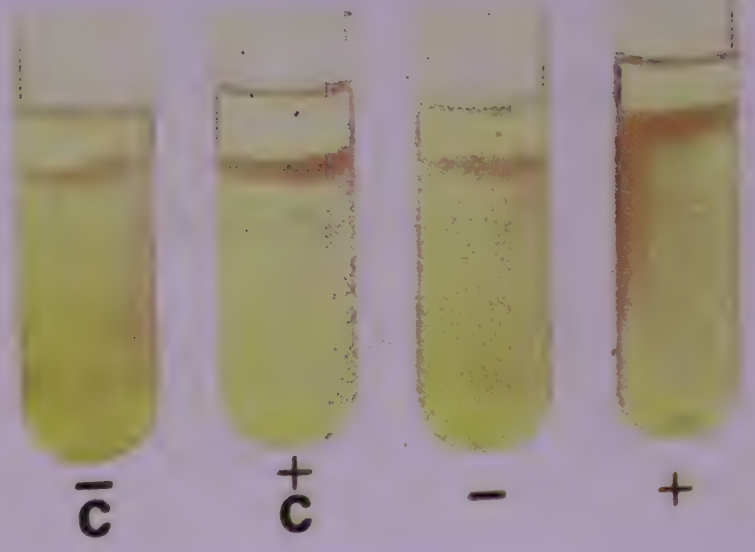
NITRATE REDUCTION TEST

B



ARYLSULPHATASE TEST

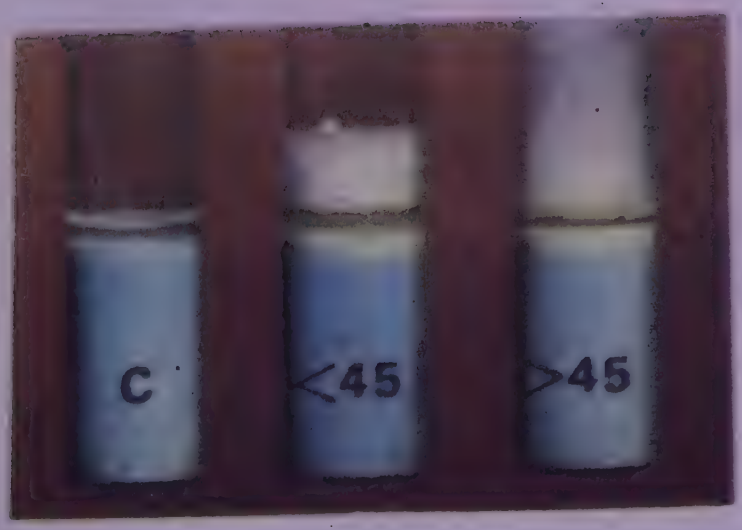
C



PYRAZINAMIDASE TEST

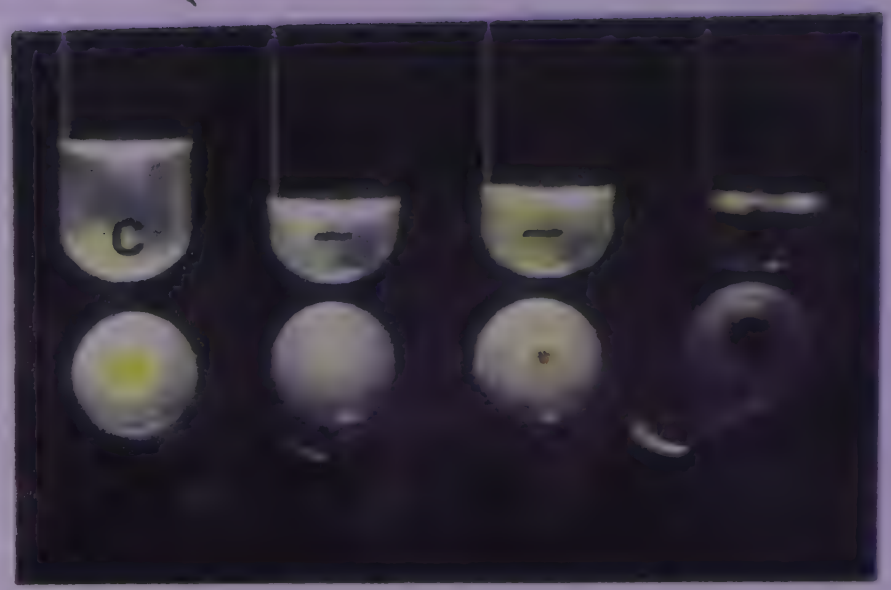
D

Fig. 14.5



SEMIQUANTITATIVE CATALASE TEST

Fig. 14.5 E



TELLURITE REDUCTION TEST

Fig. 14.5 F

4. Pape JW, Liautaud B, Thomas F, Mathurin JR, Stamand MM, Boncy M, Pean V, Pamphile M, Larouche AC and Johnson WD. Characteristics of the acquired immunodeficiency syndrome (AIDS) in Haiti. N Engl J Med. 1983; 309 : 945-950.

5. Daley CL, Chen LL, Small PM, Mugusi F, Aris E, Cegielski P, Lalinger G, Mtoni I, Mbaga IM and Murray JF. Pulmonary complications of HIV infection in Tanzania. Am Rev Respir Dis. 1992; 145 : A821.

6. Panda BN, Tewari SC, Arora PN, Jean J, Nema SK and Jayaswal R. Response of HIV seropositive tuberculosis patients to short course chemotherapy. Ind J Tub 1996; 43 : 191-194.

7. Arora VK and Kumar SV. Pattern of opportunistic pulmonary infections in HIV seropositive subjects : Observations from Pondicherry, India. Ind J Chest Dis Allied Sci. 1999; 41 : 135-144.

8. Cohn DL, Bustreo F and Raviglione M. Drug resistant tuberculosis. Review of the worldwide situation and the WHO/IUATLD global surveillance project. Clin infect Dis. 1997; 24 (suppl 1) : S 121-130.

9. Chowgule RV and Deodhar L. Pattern of secondary acquired resistance to antituberculosis drugs in Mumbai, India-1991-1995. Ind J Chest Dis Allied Sci. 1998; 40 : 23-31.

10. Garay SM and Kamelhar DL. Nontuberculous Mycobacteria in HIV infected patients. In Rom WN and Garay SM (Eds), Tuberculosis. Boston : Little, Brown, 1995. Pp 721-732.

11. Holzman RS. Infection by M.avium intrcellulare complex in the acquired immunodeficiency syndrome. In Rom WN and Garay SM (Eds). Tuberculosis. Boston : Little, Brown, 1995. Pp 711-720.

12. Horsburgh CR and Selik RM. The epidemiology of disseminated nontuberculous mycobacterial infection in the acquired immunodeficiency syndrome (AIDS). Am Rev Respir Dis. 1989; 139 : 4-7.

13. Levine B and Chaisson RE. Mycobacterium kansasii : A case of treatable pulmonary disease associated with advanced Human Immunodeficiency Virus (HIV) infection. Ann Intern Med. 1991; 114 : 861-868.

14. Theuer CP, Hopewell PC, Elias D. Schecter GF, Rutherford GS and Chaisson RE. Human Immunodeficiency Virus infection in tuberculosis patients. J Infect Dis. 1990; 162 : 8-12.

15. Forbes BA, Sahm DF and Weissfield AS (Eds). Bailey and Scott's Diagnostic Microbioogy. St. Louis : Mosby, 1998.

16. Gupta A, Sharma SK and Pande JN. Diagnostic methods for tuberculosis. Ind J Chest Dis Allied Sci. 1993; 35 (2) : 63-84.

Chapter -15

NON-MYCOBACTERIAL BACTERIAL INFECTIONS IN HIV/AIDS

15.1 Introduction

Infection due to HIV is characterised by progressive deterioration of immunologic function, generally measured by the CD4 lymphocyte count. As the CD4 lymphocyte count declines, HIV infected patients become susceptible to a variety of infections, generally termed "opportunistic", which are associated with considerable morbidity and mortality.

Although some agents like Pneumocystis carinii, Cryptococcus neoformans and Cytomegalovirus have been long recognised as principal pathogens the role of bacterial pathogens in causing morbidity related to AIDS was recognised more recently. Bacterial infections, multiple or recurrent, recurrent pneumonia and recurrent salmonella septicaemia have been included in the 1993 AIDS Surveillance Case definition.

The true incidence of non-mycobacterial bacterial infections in HIV-infected persons is difficult to discern and varies with the population surveyed. Diseases like pneumonia, sinusitis, bacteraemia and bacterial enteric infections occur at a rate many times higher than in the general population.

15.2 Magnitude of problem

The host defence defects that may predispose HIV infected patients to bacterial infections include diminished antibody response to protein and polysaccharide antigens, diminished B-cell mitogenic response, IgG subclass deficiencies, decreased production of secretary IgA, macrophage dysfunction, long-term intravenous catheterisation, encephalopathy, malnutrition, etc.

Early recognition of the onset of opportunistic infection and identification of the aetiological agent (s) associated with it are essential if treatment is to succeed. The prime concern is to institute prompt antibiotic prophylaxis treatment while pursuing the laboratory diagnosis.

HIV infected persons, especially with advanced disease, have increased susceptibility to systemic bacterial infections, in particular with encapsulated organisms (like Streptococcus pneumoniae and Haemophilus influenzae), Staphylococcus aureus and enteric gram-negative bacilli. These bacterial pathogens are often the immediate cause of death in AIDS patients and cause considerable morbidity.

Table -15.1 lists the bacteria that have been reported to cause disease with increased frequency and or of increased severity in HIV infected persons[1].

Table -15.1 Bacteria (other than mycobacteria) that cause disease with increased frequency and/ or of increased severity in HIV-infected persons, presumably because of immunosuppression

Micro-organisms	Syndrome (s)
Gram-positive bacteria	
Streptococcus pneumoniae	Pneumonia (lobar), septic arthritis, bacteraemia, sinusitis, mastoiditis, meningitis, endocarditis
Streptococcus pyogenes	Fasciitis, soft-tissue infection
Group C streptococci	Fasciitis, soft-tissue infection
Group G streptococci	Meningitis
Staphylococcus aureus	Impetigo, sinusitis, cellulitis, pyomyositis, scalded skin syndrome, septic bursitis, pneumonia, bacteraemia, osteomyelitis
Listeria monocytogenes.	Meningitis; hepatitis, bacteraemia, disseminated infection
Clostridium difficile	Pseudomembranous enterocolitis, bacteraemia
Corynebacterium jeikeium	Skin nodules, bacteraemia, disseminated infection
Corynebacterium diphtheriae	Cutaneous infection
Gram-negative bacteria	
Haemophilus influenzae	Bronchitis, pneumonia, sinusitis, otitis/mastoiditis
Salmonella species	Bacteraemia, enteritis, endocarditis, cholecystitis, septic arthritis, osteomyelitis, pneumonia (cavitary), disseminated infection
Shigella flexneri	Enteritis, bacteraemia, disseminated infection
Campylobacter jejuni	Enterocolitis, bacteraemia, ostemyelitis, endocarditis
Campylobacter fetus	Enterocolitis
Campylobacter fennelliae	Enterocolitis
Pseudomonas aeruginosa	Sinusitis, bacteramia, pneumonia, indwelling catheter infection
Pseudomonas cepacia	Osteomyelitis, bacteraemia, indwelling catheter infection
Bordetella pertussis	Pertussis
Bordetella bronchiseptica	Pneumonia, sinusitis, bronchitis
Methylobacterium extorquens (formerly Protomonas extorquens)	Bacteraemia
Legionella pneumophila	Pneumonia (cavitary), sinusitis
Neisseria meningitidis	Disseminated infection, meningitis
Kingella denitrificans	Granulomatous disease
Aeromonas hydrophila	Proctocolitis
Klebsiella rhinoscleromatis	Oropharyngeal infection (rhinoscleroma)
Bartonella (Rochalimaea) henselae	Bacillary angiomatosis, hepatosplenic infection, bacteraemia, encephalitis, lymphadenitis
Bartonella quintana	Bacillary angiomatosis, bacteramia, endocarditis
Aerobic actinomycetes	
Nocardia asteroides	Pneumonia, brain abscess, lever abscess
Nocardia brasiliensis	Liver abscess
Rhodococcus equi	Pneumonia (cavitary), pleural effusion, bacteraemia, disseminated infection
Anaerobic actinomycetes	
Actinomyces israelii	Cervicofacial infection, oesophagitis
Spirochetes	
Treponema pallidum	Primary, secondary, latent or tertiary syphilis, cutaneous infection, diarrhoea, proctitis

USPHS/IDSA Guidelines for prevention of opportunistic infections in persons infected with Human Immunodeficiency Virus [1]

Children with HIV infection are more susceptible to bacterial infections then HIV-infected adults [2]. Recurrent, serious bacterial infection is included as an "indicator disease" in the case definition of AIDS in the paediatric population. Frequently recognized clinical syndromes in children with HIV infection include:

- Pneumonia
- Otitis media
- Sinusitis
- Urinary tract infection
- Skin and soft tissue infections
- Meningitis
- Osteomyelitis
- Intravenous catheter infection

Indian Scenario

Although the number of AIDS cases is increasing in India, there is little information about OIs among these patients. One study revealed pneumococcal infection of the lung in 5% cases, S. aureus subcutaneous abscess in 5% and otitis media (S. aureus) in 3% of AIDS patients [3] while group B Salmonella septicaemia was found in 1.6 AIDS patients in a recent South Indian study [4].

15.3 Respiratory tract infections

HIV infected persons experience respiratory tract infections with a variety of bacterial pathogens including those common in non-HIV infected persons (such as S. pneumoniae, H. influenzae and Pseudomonas aeruginosa) as well as those that are typically recovered only from immuno-compromised persons (such as Rhodococcus equi). Such infections may be community or hospital acquired and may range from mild, self-limiting infections to those that are severe and life-threatening. Patients with AIDS have a markedly increased risk of acquiring Legionnaires disease also.

15.3.1 Bacterial aetiology of pneumonia/lower respiratory tract infections (LRTI) in HIV - infected persons

Common bacterial pathogens

- S. pneumoniae
- H. influenzae
- Klebsiella spp.
- Other Enterobacteriaceae
- S. aureus
- P. aeruginosa

Other bacterial agents

- Moraxella catarrhalis

- Nocardia spp.
- Rhodococcus equi
- Legionella pneumophila
- etc.

15.3.2 Clinical presentation

HIV associated respiratory disease includes upper respiratory tract infections, acute or chronic sinusitis, acute or chronic bronchitis and bacterial pneumonias. The clinical features of respiratory tract infections are the same in HIV infected individuals as in those without HIV, but their frequency is increased and there is a higher rate of complications including intra-pulmonary cavitation, abscess formation, empyema and death. HIV infected patients with bacterial pneumonia are commonly bacteraemic and may relapse after appropriate antibiotic therapy. Pneumonia is the most commonly diagnosed bacterial respiratory infection in HIV infected persons. Many of them also have one or more episodes of sinusitis during the course of their disease. Acute or chronic bronchitis is also diagnosed more frequently in HIV/AIDS patients.

Bacterial lower respiratory tract infections are manifested as cough with sputum production, variable fever, chills chest pain etc. Other symptoms may include headache, nausea and myalgias. Prompt and accurate diagnosis of the respiratory infection is essential because the outcome of HIV associated bacterial pneumonia appears to be reasonably good with appropriate treatment.

15.3.3. Laboratory diagnosis of LRTI (Pneumonia)

The bacterial agents that must be sought depend upon the available expertise, facilities and resources. Microscopy alone may be performed at primary/peripheral level health care setting, while culture of common pathogens may be possible at the intermediate/district health centres. Isolation of fastidious bacterial agents should be attempted only in tertiary level hospital laboratories. Rapid, simple, non-culture methods for the detection of bacterial antigens or antibodies offer a better alternative to culture at primary and intermediate health care facilities, provided they are reasonably sensitive, specific and economical. Bacterial isolates may be submitted to tertiary/referral centres for further characterizaton by serotyping, etc, besides, antimicrobial susceptibility testing. This is essential for designing prophylaxis by vaccination or antibiotic therapy. Tertiary health care facilities that have adequate resources and trained personnel may undertake diagnosis by the more sensitive and specific molecular methods like PCR and DNA hybridisation etc., primarily to evaluate other methods like microscopy and culture.

Specimen collection and transport

Sputum

- Deep coughed sputum should be expectorated directly into a sterile, wide mouth container. Sputum production may be assisted by using postural drainage and thoracic percussion.
- In patients who are unable to produce sputum, aerosol induced sputum may be collected by allowing the patient to breathe aerosolised droplets of a solution containing 15% sodium chloride and 10% glycerin for 10 minutes.

Bronchial aspirate, broncho-alveolar lavage (BAL)

During bronchoscopy, physician may obtain bronchial washings or aspirates and broncho-alveolar lavage samples.

- Bronchial washings obtained by instilling a small amount of sterile saline into the bronchial tree and withdrawing the fluid.
- Broncho-alveolar lavage specimens: 100–300 ml of sterile saline is infused into a lung segment to obtain cells and secretions from the alveolar spaces and pulmonary interstitium.

Blood for culture

See 15.4 for method of collection, transport and processing

Specimen processing

Direct examination

Preparation of smears for staining

A portion of the sputum specimen consisting of purulent material is used to prepare smears for gram staining and staining by other methods. Other respiratory specimens like BAL, etc. need to be concentrated by centrifugation and the sediment is used for preparation of smears and inoculation of culture media.

Staining method

Gram stained smear examination is first done to evaluate the quality of the respiratory sample collected. An acceptable sputum specimen is one that shows less than 10 squamous epithelial cells per low power fileld (100 x). The number of white blood cells (WBCs) may not be relevant, but the presence of 25 or more WBCs per 100 x field indicates an excellent specimen. The presence of ciliated columnar bronchial epithelial cells, goblet cells or pulmonary macrophages in the bronchial aspirate or BAL indicates a specimen from the lower respiratory tract.

Presumptive aetiologic diagnosis by gram staining is possible if there are numerous WBCs/ neutrophils along with :

- A predominance of gram positive diplococci which is suggestive of pneumococcal pneumonia. (Fig. 15.1)
- A predominance of gram negative coccobacilli which is suggestive of H. influenzae iniection.
- Demonstration of gram positive branching or partially branching, beaded filaments which is suggestive of infection with Nocardia spp. (Fig. 15.2)
- Presence of diphtheroid like gram positive coccobacilli or rods with minimal branching which is suggestive of Rhodococcus infection.

Modified acid fast staining

Witn 1 % sulphuric acid as decolourising agent : Presence of pink/violet, branching, fine filaments with fragmentation, bacilli, coccobacilli indicates presence of partially acid fast actinomycetes like nocardia and rhodococcus.

Other staining methods to detect Nocardia and other actinomycetes include

- Gomoris methanamine silver stain and toluidine blue O stain. (Chapter 13)

Direct fluorescent antibody (DFA)

- DFA staining can be done for detection of Legionella pneumophila in lower respiratory tract specimens.

Culture

Most of the commonly sought aetiologic agents of pneumonia like S. pneumoniae, H. influenzae, P. aeruginosa, S. aureus and streptococci can be isolated on routinely used media like 5% sheep blood

agar, MacConkey agar and chocolate blood agar. For Legionellae the specimen should be inoculated on buffered charcoal-yeast extract agar (BCYEa) and selective BCYEa. Numerous bacterial agents that cause pneumonias are not detected by routine bacteriologic culture and require special procedures for detection.

Prolonged inoculation of routine media like sheep blood agar and chocolate agar for upto 7 days is required for growth of Nocardia spp. and Rhodococcus spp., but may be overgrown by contaminating flora, hence selective media have been developed.

Table -15.2 shows the cultural and morphological characteristics of some of the major bacterial agents associated with pneumonia in HIV infected persons.

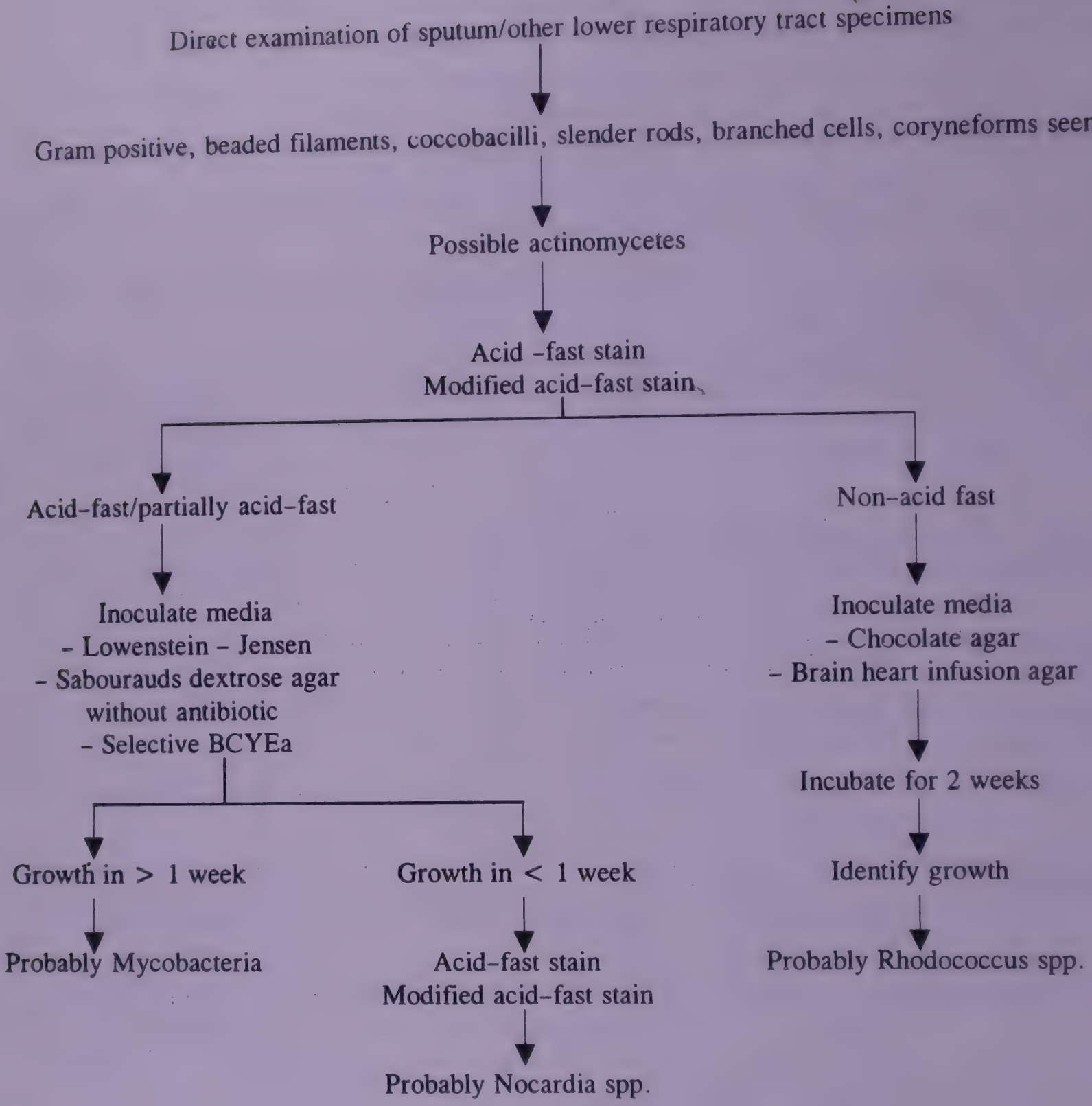
Growth and morphology of some respiratory bacterial pathogens recoverd from HIV infected persons with pneumonia

Bacterium	Isolaton media	Colony characteristics	Morphology on Gram stain
S. pneumoniae	Sheep blood agar	AH, 0.5-1mm, smooth, transparent, low convex/flat/ centrally depressed	GP diplococci, short chains
H. influenzae	Chocolate agar	0.5-1mm, round, low convex, smooth, transparent, fishy seminal odour	Small GP rods or coccobacilli
S. aureus	Sheep blood agar	BH/NH, 1-3mm, smooth, rough, opaque, cream to gold pigmented	GP cocci in grape like clusters
P. aeruginosa	Sheep blood agar	Large, irregular, with diffuse haemolysis	GN bacillli
	MacConkey agar	Pale (NLF) large, irregular, grape-like odour	
Rhodococcus equi	Sheep blood agar Chocolate agar Selective BHIA	NH, mucoid, originally white, later salmon-pink pigmented	GP coccobacilli, diphtheroid/ coryneform
Nocardia spp.	Sheep blood agar Sabourauds Dextrose agar Lowenstein-Jensen medium Paraffin agar Selective BCYEa	Variable, some BH, dry-chalky, heaped-folded, Gray-white to yellow/orange tan with musty odour	GP fine, branching filaments
L.pneumophila	BCYEa Selective BCYEa	1-3 mm, circular, convex, gistening	GN bacilli

GP = Gram positive
GN = Gram negative
NLF = Non-Lactose Fermenter
BHIA = Brain Heart Infusion agar
BCYEa = Buffered Charcoal Yeast Extract agar

AH = Alpha-haemolytic
BH = Beta-haemolytic
NH = Non-haemolytic
GN = Gram negative

Flow chart 15.1



15.4 Bacteraemia

Bacteraemia may indicate the presence of a focus of infectious disease or may merely represent transient release of bacteria into the blood stream. Bacteraemia (presence of bacteria in the blood stream) may be transient, continuous or intermittent. Causes of continuous bacteraemia include septic shock, bacterial endocarditis, early stage of typhoid fever, brucellosis, leptospirosis, etc. Intermittent bacteraemia occurs in meningitis, pneumonia, osteomyelitis, undrained abscesses etc.

Septicaemia indicates a situation in which bacteria or their products (toxins) are causing harm to the host. The clinical manifestations of septicaemia may include fever, chills, hyperventilation, skin lesions and lead to hypotension or shock, major organ system failure and disseminated intravascular coagulation (DIC).

Blood stream infections may be

- Intra-vascular : eg., infective endocarditis, intravenous cathetar associated infections.
- Extra-vascular : eg., bacteria enter the blood stream from the primary extra-vascular focus of infection via the draining lymphatic system.

In most cases of bacteraemia in HIV infected patients, a primary focus of infection is identified as a source of bacteraemia. The best recognised associations include Streptococcus pneumoniae bacteraemia secondary to pneumonia, non-typhoidal salmonella spp. bacteraemia secondary to gastro-intestinal disease and S. aureus bacteraemia secondary to intravascular cathetar infections. Nosocomial bacteraemias in which gram negative bacilli and staphylococci are isolated also occur and typically result from intra-vascular cathetar infections, pneumonia or infections of the urinary tract.

Patients with AIDS have the greatest diversity of bacterial agents recovered from blood including

- Staphylococcus aureus
- Enterococci
- Coagulase negative staphylococci
- Pseudomonas aeruginosa
- Enterobacteriaceae
- Shigella flexneri and other shigella spp.
- Salmonella spp. (besides S. typhi and S. paratyphi)
- Bartonella spp.
- Corynebacterium jeikeium
- Listeria monocytogenes etc.

Detection of bacteraemia

Specimen collection

- The skin site over the chosen vein is cleaned with 70% alcohol and an antiseptic.
- Gloves must be worn for drawing blood (as part of the universal precautions).
- The top of the blood culture bottle is disinfected before introducing the blood sample through the same.

Specimen volume

- Adults - 10 ml
- Children - 1-5 ml

Number of specimens

2 or 3 blood samples collected on the same day or at the same time from different sites is usually sufficient.

Blood culture media

50 ml for adults, 25 ml for children of basic blood culture media containing nutrient broth, trypticase soy broth, Brain Heart Infusion broth etc with or without an anticoagulant like sodium polyanethol sulphonate (0.025-0.05%).

Transport

The inoculated blood culture bottles are transported to the laboratory at room temperature within 2 hours of collection. In case of delay beyond 2 hours, they are to be incubated at 35–37°C prior to transport at ambient temperature.

Laboratory procedures

The blood culture bottles are incubated aerobically at 35–37°C and blind subcultures performed, first after 6–18 hours of incubation and subsequently after every 48 hours upto 5–10 days. The number of blind subcultures performed varies from 3–5 for routinely isolated organisms. Blind subculture is performed aseptically by removing a few drops of the well–mixed medium and spreading onto solid media like chocolate blood agar, 5% sheep blood agar and MacConkey's agar.

The blood culture bottles may also be examined visually daily for macroscopic evidence of growth which is indicated by development of :

- Haemolysis of RBCS
- Gas bubbles in the medium
- Turbidity
- Small aggregates of bacterial growth in the broth or in the sedimented RBC layer.

When macroscopic evidence of growth is seen, a smear is prepared from a drop of the medium and subjected to gram staining after methanol fixation.

Isolation of fastidious, rarely isolated organisms requires different and special conditions for their successful recovery from blood culture samples. If these are suspected then the laboratory should be alerted to hold the blood cultures beyond the usual duration of 7–10 days and make blind subcultures onto several enriched media.

An isolate may be considered as a probable contaminant if :

- Bacillus spp., Corynebacterium spp. or coagulase negative staphylococci is isolated in only one of several cultures from a patient.
- There is growth of multiple organisms from only one of several cultures.
- The organism causing infection at the primary site is not the same as that isolated from blood culture.

An isolate may be considered as a probable pathogen in case there is

- Growth of the same organism in repeated blood cultures obtained at different times or from different sites.
- Growth of enterobacteria; S. pneumoniae, S. pyogenes, H. influenzae etc.
- Growth of commensal microflora from blood cultures obtained from patients suspected to be bacteraemic, eg. immuno–compromised patients.

15.5 Bacterial enteric infections in HIV infected persons

Several bacterial enteric infections occur with increased frequency in persons infected with HIV and some of these are more likely to be severe, recurrent, persistent and associated with extra–intestinal disease

Enteric pathogens recovered from HIV infected persons include

- Shigella flexneri, other Shigella spp.
- Salmonella spp.
- Campylobacter spp.
- Enterohaemorrhagic E. coli
- Enteroinvasive E. coli
- Clostridium difficile
- Vibrio cholerae
- S. aureus
- Plesiomonas shigelloides
- Aeromonas hydrophila
- Yersinia enterocolitica
- etc.

15.5.1 Magnitude of problem

Salmonella infections

Salmonellosis is likely to cause severe invasive disease in HIV infected persons and antimicrobial therapy is recommended to prevent extra-intestinal spread. Salmonella septicaemia may manifest like typhoid fever, with few gastro-intestinal symptoms. CDC survillance definition has recently included recurrent non-typhoidal salmonella septicaemia (RSS) as an AIDS defining illness. Not all serotypes of salmonella are equally likely to produce RSS. Some serotypes are "opportunistically invasive", i.e., cause invasive illness in immuno-compromised individuals, e.g. Salmonella typhimurium, S. enteritidis, S. dublin. However, different serotypes may be associated with RSS in different geographic areas. .

Shigella infections

The incidence of Shigella flexneri infections among young adult men has increased, among male homosexuals in the US. However, there is not much increased risk of shigellosis in HIV infected persons. Shigella bacteraemia, a rare complication of shigellosis in adults, may be more common in HIV infected patients.

15.5.2 Laboratory diagnosis of diarrhoea

Diarrhoea is experienced by over 50% of patients with AIDS and can be a major source of morbidity and mortality. Specific pathogens may be isolated in upto 75-80% of patients in developed countries, with bacterial causes being identified in approximately 20% of cases. Stool analysis using three samples will identify majority of the aetiologic agents, the frequency of "pathogen-negative" diarrhoea depends upon the extent of diagnostic investigations undertaken. In patients with HIV related diarrhoea the minimal evaluation should consist of a careful search for routine bacterial enteric pathogens by culture and examinaton of the stool for C. difficile toxin if the patient has received antibiotics within the past 2-3 months. Enteric bacteria are usually detected by routine stool cultures but are occasionally found only in the blood.

Specimen collection and transport

A freshly passed faecal stool sample should be collected in a clean, waxed, carboard or plastic container. About 1 teaspoon of liquid stool or pea sized amount of formed stool is sufficient. The sample should be delivered to the laboratory with in 1–2 hours of collection, if delay beyond 2 hours is expected, the specimen should be placed in a transport medium (Cary–Blair medium or buffered glycerol saline) and kept at 4^0 C until it can be processed. In case stool is unavailable, a rectal swab may be collected and transported in Cary–Blair medium containing reduced agar (1.6 g/ litre).

Direct examination

Wet mount microscopy is useful for detection of faecal leucocytes which are mostly present in gastro–enteritis due to shigellae, non–typhoidal salmonellae, Campylobacter and entero–invasive E. coli but may or may not be present in infections due to Vibrio cholerae, C. difficile, Plesiomonas shigelloides and entero–haemorrhagic E. coli. Examination of wet mounts under darkfield or phase contrast may reveal the darting motility and curved forms of Campylobacter spp.

The use of Loefflers methylene blue instead of saline for preparation of a wet mount is preferred in some laboratories to visualise faecal leucocytes.

Gram staining of faecal smears : presence of numerous thin, comma shaped gram negative bacilli may indicate presence of infection due to Campylobacter or Vibrio spp. Faecal leucocytes could also be visualised in gram stained smears.

The use of carbol fuchsin or basic fuchsin as counterstain for gram staining is preferred for better visualisation of Campylobacter organisms in smears of stool.

Culture

Stools received for routine culture should be examined for Salmonella spp., Shigella spp., Campylobacter spp., E. coli (enteroinvasive/enterohaemorrhagic) and C. difficile. The organisms sought should take into account the incidence of gastrointestinal tract infections caused by particular aetiologic agents in the area.

Isolation and identification of Yersinia enterocolitica, Plesiomonas, etc. may be included if resources and facilities permit the same.

For routine culture stool specimens should be plated onto

- A slightly inhibitory and differential medium, e.g. MacConkey agar
- A moderately selective medium, e.g. Xylose - Lysine Deoxycholate agar (XLD) or Deoxycholate Citrate Agar (DCA)
- Enrichment broth to enhance the recovery of low numbers of salmonellae and shigella spp. e.g. selenite or Gram Negative (GN) broth
- Cultures for isolation of Campylobacter jejuni should be inoculated on a selective agar containing antimicrobial agents that suppress the growth of normal flora, e.g. blood–free charcoal cefoperazone deoxycholate agar (CCDA) and blood containing Skirrows medium.

Thiosulphate – Citrate – Bile salts – Sucrose (TCBS) agar is the selective – differential medium of choice for the recovery of pathogenic vibrios from stool.

Enterohemorrhagic E. coli may be detected by inoculating faecal samples into Sorbitol MacConkey agar, which is a useful selective and differential screening medium.

Incubation

Media for isolation of Campylobacter are to be incubated at 42°C for upto 72 hours in a microaerophilic environment.

All other culture media are incubated in air at 37°C for 48 hours and examined daily.

On MacConkey agar all non-lactose fermenting colonies should be subjected to further tests for identification of the bacterial isolate(s).

C. difficile toxin assay

- 10-20 ml of watery stool is collected and may be stored at 4°C for upto 3 days, but should be frozen at -70° C if a longer delay is anticipated.
- Freezing at -20°C is contraindicated as this results in a dramatic loss of cytotoxin activity. Swab specimens are non suitable.
- Culture of the organism from faeces is not considered diagnostic, because a significant proportion of the adult population carries C. difficile.
- The current methods for detecting C. difficile associated disease include:
  - Enzyme immunoassay for toxins A and B.
  - Latex agglutination assays for toxins A and B.

These are reasonably sensitive and easy to use but vary in their specificity. The kit to perform these assays are available commercially.

15.6 Central nervous system infections

Infections of the CNS usually occur as part of the disseminated disease. The onset is insiduous and infection is suspected at an advanced stage. The clinical manifestations are fever, headache, stiff neck, nausea and vomiting. The major causes of CNS infection in HIV infected persons may be conventional organisms or unusual agents of meningitis including established opportunistic pathogens.

- S. pneumoniae
- S. aureus
- Listeria monocytogenes
- Various gram-negative bacilli
- Nocardia spp.
- Treponema pallidum
- Actinomyces spp.
- etc

Clinical specimens: Blood and CSF

Specimen processing

CSF examination should include :

- Microscopy of centrifuged sediment
- Antigen detection tests using commercially available kits
- Culture for conventional and unusual pathogens
- CSF. should also be examined for cell counts, glucose and protein levels to differentiate between bacterial and aseptic/viral meningitis.

15.7 Brief description of some of the bacterial species that are more likely to be associated with opportunistic infections in HIV/AIDS is given below :

15.7.1 Nocardia spp.

Clinical specimens

Lower respiratory tract specimens (sputum, bronchial washings/lavages etc.) CSF, blood, exudates and tissues

Note : Samples suspected of containing Nocardia should not be refrigerated prior to plating, since some strains may lose viability.

Direct examination

Gram positive, beaded filaments showing branching, short filaments, rods, cocci or coryneform rods.

Modified acid fast stain positive organisms of similar morphology

Culture

Nocardia spp. are aerobic organisms that can grow on ordinary media like blood agar, Brain Heart Infusion agar and Sabourauds dextrose agar without antibiotics, incubated at 35–37°C in air or 5–10% $C0_2$ for 2–6 weeks. Can also grow on modified Thayer–Martin medium and selective BCYEa. Paraffin agar serves as an inexpensive selective medium for isolation of Nocardia.

Nocardia asteroides can survive the N–acetyl cystiene digestion procedure (without NaOH) used for sputum pre–treatment and some strains also grow on media used for primary isolation of Mycobacteria e.g. 7H10, 7H11 or Lowenstein–Jensen medium.

Colony characteristics (Fig. 15.3)

Extremely variable, some strains are ã–haemolytic on sheep blood agar. Typical colonies of Nocardia spp. are dry to chalky in consistency, heaped or folded and range in colour from yellow – orange to grey–white with a pungent musty–basement odour. On LJ medium colonies develop within 1–2 weeks and may be similar to atypical mycobacteria

Identification

Gram stain of suspected colony shows gram positive delicate, branching filaments

- Modified acid fast stain : Nocardia are partially acid–fast – i.e. they do not decolourise when treated with 1% H_2SO_4 in the Ziehl – Neelsen stain (Fig. 15.4)
- Growth in Nutrient broth with lysozyme is useful in identifying Nocardia species, especially those that are non–acid fast
- Presence of aerial hyphae

15.7.2 Rhodococcus equi

Clinical specimens : Lower respiratory tract specimens, blood, cathetar tips, etc.

Direct examination

- Gram – positive short rods or coccobacilli, often in pallisades
- Small percentage of strains are partially acid fast (1% H_2SO_4)

Culture

Grows well on routine media like sheep blood agar and chocolate agar, but not on MacConkey agar. Rate of growth is slow, hence plates should be sealed to prevent drying. Incubation in air or 5% carbon dioxide is recommended for 3 weeks. Plates are examined every 48 hours for 1 week and every 96 hours for two further weeks.

Selective medium used to prevent overgrowth by normal microflora in respiratory samples e.g.

- Brain Heart Infusion blood agar with chloramphenicol and cycloheximide
- Columbia agar with Colistin and Nalidixic acid

Colony characteristics (Fig. 15.5)

Originate as clear to white, non-hemolytic, round colonies that are often mucoid with salmon-pink pigment developing in 4–7 days (colour may also vary from white, cream, red, orange to yellow).

Tests for identification

Gram stained smear from colonies shows gram positive coccobacilli, in zig-zag configuration (diphtheroid like), with minimal branching (Fig. 15.6).

- Non-motile
- Catalase-positive
- Urease-positive
- Oxidase-negative
- Aerial hyphae not produced on tap water agar
- No growth in nutrient broth with lysozyme
- Nitrate reduction positive mostly

15.7.3 Legionella pneumophila

Clinical specimens

Expectorated sputum, materials collected during bronchoscopy (BAL, washings etc.), closed and open lung biopsy material, fine needle aspirates of lung, pleural fluid

Direct examination

Direct smears of exudates or touch preparations (dab smears) of lung biopsy material should be fixed with methanol and subjected to

- Gram staining*
- Giemsa staining
- Direct fluorescent antibody (DFA) procedure

* The intensity of staining can be improved by staining with safranine for 10 minutes or by adding 0.05% carbol fuchsin to safranine.

DFA (Direct fluorescent antibody staining)

DFA has a sensitivity of 25–70% and a specificity of 95%. Specific antibody in the form of FITC labelled polyvalent antiserum and other reagents required for Legionella DFA testing can be

purchased commercially. The clinical specimen is fixed on a slide and overlaid with FITC labelled antibody. The antigen present on or in Legionella organisms in the patient specimen binds the FITC labelled antibody, and the bacteria can be seen as brilliantly fluorescing yellow-green rods under ultraviolet light using a fluorescent microscope.

Culture

Aerobic and fastidious, does not grow on routinely used media like sheep blood agar etc, but may grow slowly on chocolate agar used for gonococcus isolation. Non-selective solid medium used for isolation of Legionellae is buffered charcoal yeast extract agar (BCYEa). Use of one or more BCYEa based selective media is recommended to avoid overgrowth by normal flora. Plates are incubated in 5-10% CO_2 at 35°C and examined every 2-3 days for upto 2 weeks.

Acid wash decontamination of sputum prior to inoculation of isolation media aids in the inhibition of normal flora and improves the isolation of Legionellae.

Colony characteristics

On BCYEa - variable size (1-3 mm), glistening, convex, circular (Fig. 15.7)

Identification

- Gram stained smear from colony shows Gram-negative bacilli (Fig. 15.8)
- Colonies suspected of being Legionellae should be subcultured onto 5% sheep blood agar or L-cystiene deficient BCYEa. Organisms that do not grow on these media are probably Legionellae.
- The most convenient method for confirming a suspected Legionella isolate is the DFA test.
- An isolate with morphological growth properties similar to Legionella that does not give a positive result by the DFA test should be sent to a reference laboratory for confirmation.

15.7.4 Listeria monocytogenes

Clinical specimens: CSF, blood, stool

Direct examination

In gram-stained smear of CSF short, gram-positive bacilli, coccobacilli, occasionally diplobacilli in short chains may be seen intracellularly or extracellulary. These may be mistaken for pneumococci.

Culture

Can be grown on sheep blood agar (tryptic soy agar base) incubated at 35° C for 24 hours in air or 5-10% carbon-dioxide

Selective enrichment for stool specimen

- Cold enrichment in non-selective broth at 4°C for 2 months and then plating on selective medium.
- Lithium chloride - Phenyl ethanol - Moxalactam agar (LPM medium)

Colony characteristics (Fig. 15.9)

Colonies on sheep blood agar are small, translucent, gray surrounded by a narrow zone of â-haemolysis (often mistaken for Group B streptococci).

Identification tests

- Gram staining - short gram-positive bacillli, coccobacilli (Fig. 15.10)
- Catalase test positive
- Optimal motility at 25°C (motility at 37°C is absent or very sluggish)
- Hydrolysis of esculin.

15.7.5 Corynebacterium jeikeium

Clinical specimen: Blood

Direct examination : Short, gram positive bacilli in small diphtheroid-like clusters (Fig. 15.11)

Culture

- Can grow well on sheep blood agar
- Plates should be incubated in air or 5-10% carbon-dioxide for 48 hours.

Colony characteristics : Punctate, smooth, white colonies (Fig. 15.12)

Identification

- Gram staining of smears prepared from colony : pleomorphic gram-positive rods that vary from short coccobacilli to long bacilli.
- Lipid-requiring - produces turbid growth in Brain Heart Infusion broth with 1% Tween 80 and no visible growth in unsupplemented medium.
- Nitrate reduction negative
- Urease negative.
- Acid produced from glucose
- Resistant to most antibiotics
- Susceptible to vancomycin

15.7.6 Campylobacter spp.

Clinical specimens : Stool, blood, etc.

Direct examination

In gram-stained preparations of diarrhoeal stool presumptive diagnosis of Campylobacter enteritis is made by observing gram negative bacilli that are curved, s-shaped or long spiral forms (Fig. 15.13)

Culture

Sucessful isolation of C.jejuni/coli from stool depends upon :

- Use of selective media e.g. - Campy - BAP, Skirrows blood agar, blood-free charcoal-based selective media.
- Incubation at 42°C in an atmosphere containing 5% oxygen, 10% carbon-dioxide and 85% nitrogen.

Colony characteristics (Fig. 15.14, 15.15)

Flat, gray, irregular colonies that may be dry or moist, or round, convex, glistening.

Identification

- Appearance of colonies on the selective medium that has been incubated at 42^0 C indicates that the organism is a thermophilic Campylobacter species, most commonly C.jejuni
- Catalase positive
- Oxidase positive
- Hippurate hydrolysis : positive in C.jejuni and negative in C.coli

15.7.7 Bartonella spp.

Bartonella spp. (B.henselae, B. quintana) are primarily associated with infections in the immuno-compromised host, particularly in patients with HIV infection. Infections produced include:

- Bacillary angiomatosis (cutaneous or systemic)
- Peliosis hepatis
- Fever with bacteraemia
- Endocarditis

These may represent manifestations of cat-scratch disease (CSD) in the immuno-compromised host. CSD is a common cause of lymphadenopathy in children and adolescents, 90% of whom have a history of exposure to cats, most often kittens under 1 year of age.

Clinical specimens: Blood, biopsy tissue (skin, lymph node, liver and spleen)

Direct examination

- Warthin-Starry Silver staining of tissue sections may reveal numerous interstitial bacilli, suggestive of Bartonella.
- Tissue biopsies may also be stained with immunofluorescence or immuno-histochemical technique to detect presence of the organisms.

Culture

- The organism grows best on chocolate agar and Columbia agar with 5% sheep blood.
- Incubation at 35-37^{0}C in 5% carbon dioxide is recommended for upto 30 days.
- For isolation of Bartonella spp. from blood, direct plating of blood or plating of lysis-centrifugation processed blood is required. Tissue is to be homogenised and directly plated.

Colony characteristics

Visible colonies usually appear in 5-15 days after incubation. B.henselae colonies are white, dry adherant, cauliflower', like, embedded in the agar and morphologically heterogenous.

Identification

Gram staining of colonies reveals small, gram-negative, slightly curved bacilli that are :

- Oxidase negative
- Catalase negative

- B.henselae, the most common species, shows 'twitching' motility when mounted in saline, and is urease negative.
- Confirmatory identification of species requires gas liquid chromatography of cellular fatty acids or use of B.henselae fluorescent antibody staining.

Serology

Immunofluorescence/Enzyme Immuno-assay based serological assays for detection of IgG, IgM and IgA antibodies have been developed and found helpful in making diagnostic and therapeutic decisions.

15.8 Sourcing of media, reagents & chemicals

1. HiMedia Laboratories Ltd.
   A-406, Bhaveshwar Plaza
   LBS Marg, Mumbai - 400086.
   Tel : 91-22-5000970, 5001607
   Fax : 91-22-5005764, 5002468

2. BBL - Difco :
   Becton Dickenson India Pvt. Ltd.
   The Surya, Best Western Hotel 'E' Floor
   New Friends Colony
   New Delhi - 110065.
   Tel : 91-11-6831192, 6830987
   Fax : 91-11-6831783, 6824056

3. Argene-Biosoft products marketed in India by :
   Spectral Diagnostics Pvt. Ltd.
   C-32, Kirti Nagar,
   New Delhi - 110015.
   Tel : 91-11-5931105, 5195623
   Fax : 91-11-5458049

4. Vibles Biotech
   Vibles Bhawan, 297/14
   Faridabad - 121007
   Tel : 0091-8-283416
   Fax : 0091-11-575500

5. Oxoid Culture Media
   Qualigens Fine Chemicals
   Glaxo India Ltd.
   Dr. Annie Besant Road, Worli
   P.B. No. 29113
   Mumbai - 400025.
   Tel : 022-4933871/3514
   Fax : 022-4935388/4962573

15.9 References :

1. USPHS/IDSA Guidelines for prevention of opportunistic infections in persons infected with Human Immunodeficiency Virus. JE. Kaplan, et. al. Clinical Infect. Dis. 1995; 21 (suppl.1) SI-11.

2. Management of the HIV-infected patient. Part 1 & II. J.W.M. Gold, EE Telzak, DA White. The Medical Clinics of North America. Volume 80 (6) Nov., 1996.

3. Spectrum of opportunistic infections among AIDS patients in Tamil Nadu, India. N. Kumaraswamy, Suniti Solomon, S.A. Jayaker Paul, R. Venilla, R.E. Amalraj. Int. J. of STD & AIDS 1995; 6 : 447-449.

4. Clinical and Laboratory Profile of Sixty Patients with AIDS : A South Indian Study. J. George, A. Hamide, A.K. Das, S.K. Amarnath & R. Sambasiva Rao. South-East Asian J. Trop. Med. Public Health. 1996; 27 (4) : 686-691.

5. Mackie & McCartney " Practical Medical Microbiology" Ed. JG. Collee, AG. Fraser, B.P. Marmion and A. Simmons 14 th Ed., 1996. Churchill Livingstone, N.Y., USA.

6. Bailey and Scott's "Diagnostic Microbiology". B.A. Forbes, D.F. Sahm, A.S. Weissfeld 10th Ed., 1998. Mosby., Inc.

7. "Manual of Clinical Microbiology". Ed. P.R. Murray 6th Ed. 1995. ASM Press, Washington DC, USA.

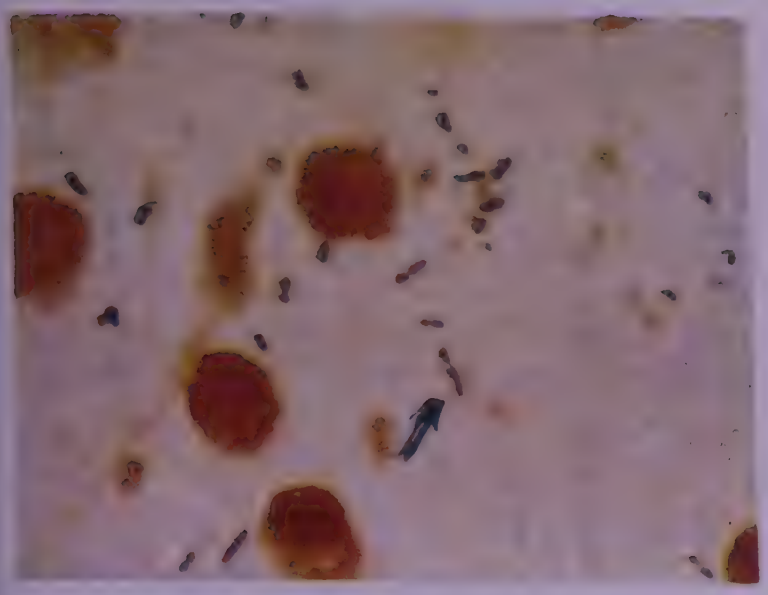


FIG. 15.1 SPUTUM SHOWING WBC'S AND GRAM POSITIVE DIPLOCOCCI (PNEUMOCOCCI).

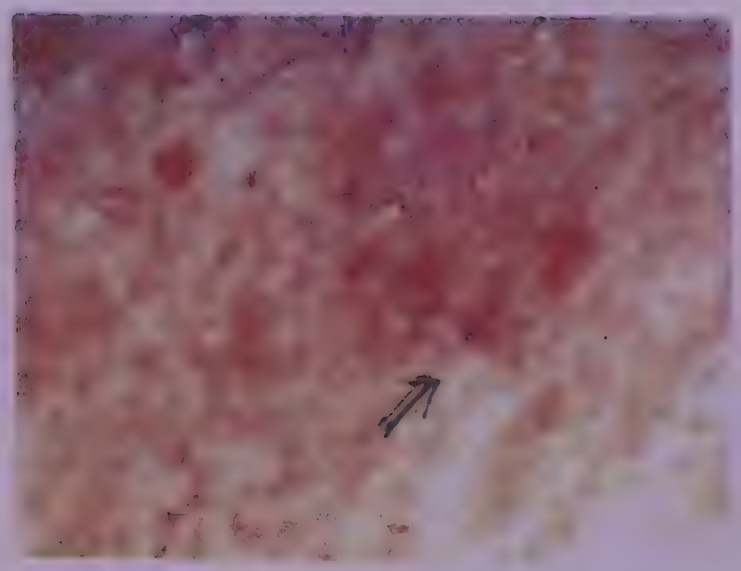


FIG. 15.2 NOCARDIA SPP. IN GRAM STAINED SMEAR .

FIG. 15.3 GROWTH OF NOCARDIA ASTEROIDES ON MIDDLEBROOK 7 H 11 AGAR.

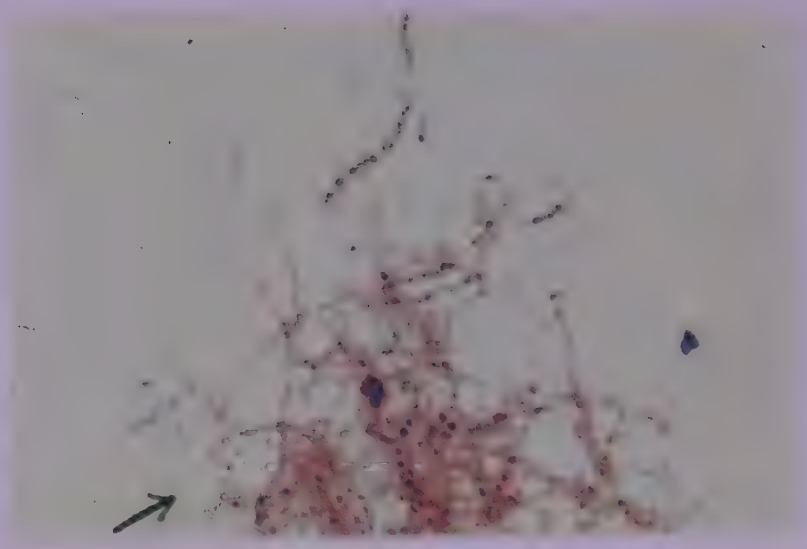


FIG. 15.4 MODIFIED ACID-FAST STAIN SHOWING ACID FAST, BRANCHING FILAMENTS OF NOCARDIA SPP.

FIG. 15.5 GROWTH OF RHODOCOCCUS SPP. ON CHOCOLATE AGAR.

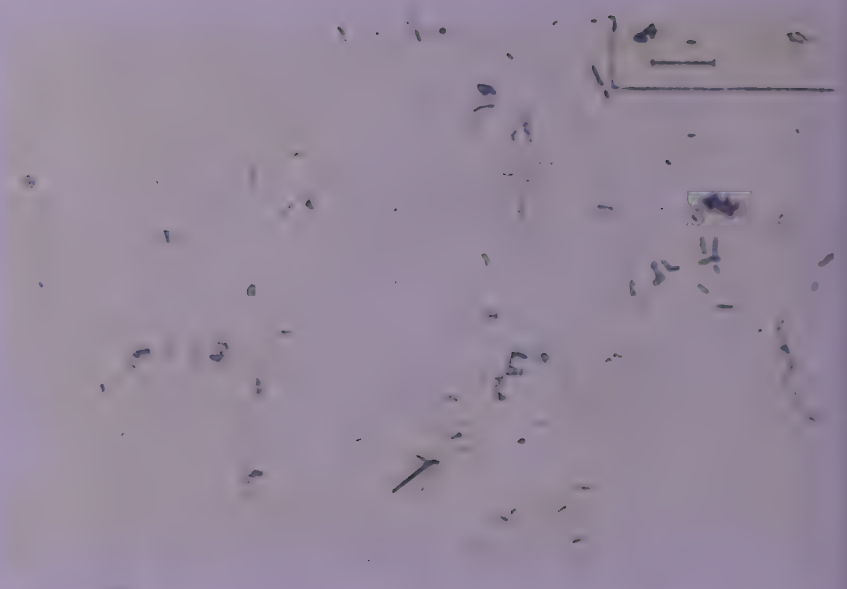


FIG. 15.6 GRAM STAIN OF RHODOCOCCUS SPP.

FIG. 15.7 GROWTH OF LEGIONELLA ON BCYE AGAR.

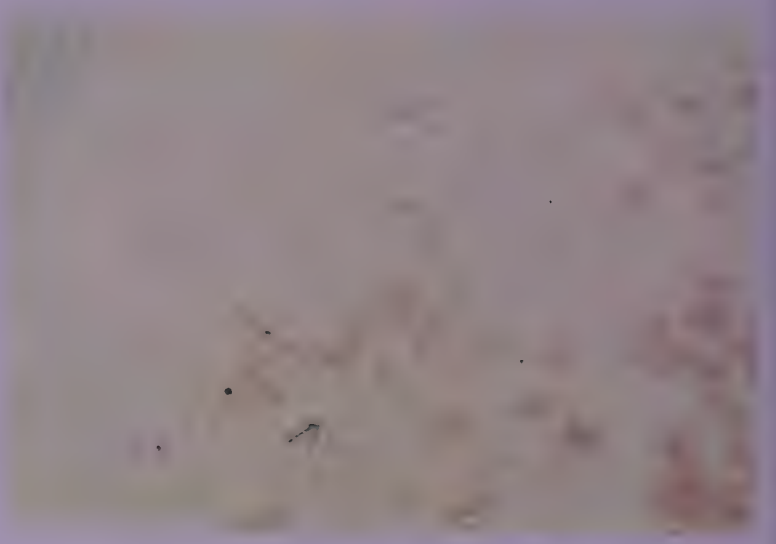


FIG. 15.8 GRAM STAIN OF LEGIONELLA PNEUMOPHILA.

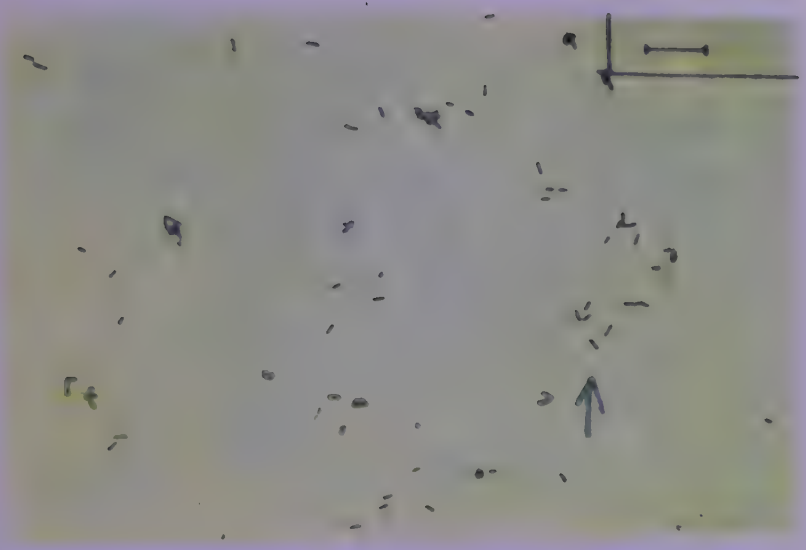


FIG. 15.9 GRAM STAIN OF LISTERIA MONOCYTOGENES.

FIG. 15.10 GROWTH OF LISTERIA MONOCYTOGENES ON SHEEP BLOOD AGAR.

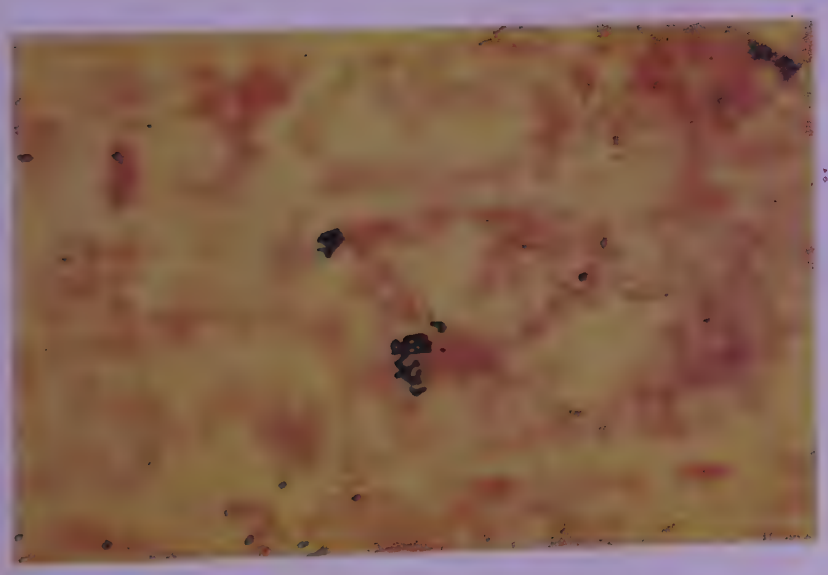


FIG. 15.11 GRAM STAIN OF C.JEIKEIUM FROM BLOOD CULTURE.

FIG. 15.12 COLONIES OF C.JEIKEIUM ON SHEEP BLOOD AGAR.

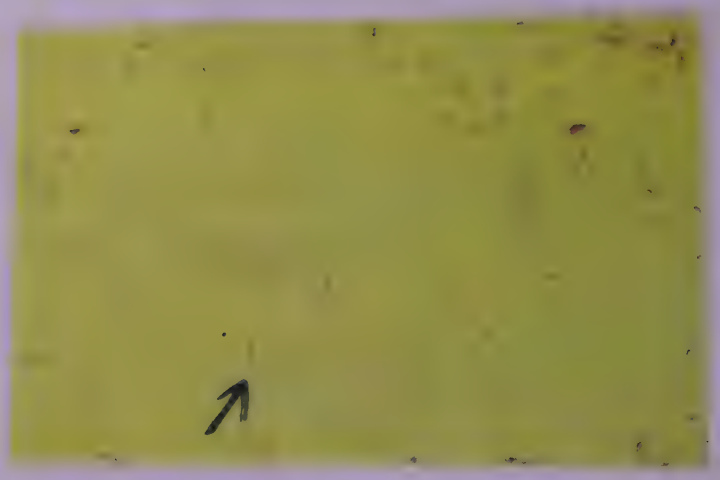


FIG. 15.13 GRAM STAIN OF CAMPYLOBACTER JEJUNI.

FIG. 15.14 GROWTH OF C. JEJUNI ON BLOOD AGAR.

FIG. 15.15 GROWTH OF C. JEJUNI OF CAMPY - BAP. AGAR.

SECTION –V : VIRAL INFECTIONS

Chapter –16

OPPORTUNISTIC VIRAL INFECTIONS IN AIDS PATIENTS

16.1 Introduction

Viral infections are very common in HIV infected individuals, especially those viruses where cell mediated immunity plays a major role in host defence. These include Cytomegalovirus (CMV), Epstein Barr virus (EBV), Herpes simplex virus 1&2 (HSV –1&2), human Herpes virus 6 (HHC–6), Varicella Zoster virus (VZV) and some of the Polyoma, Papilloma, Adeno and Respiratory viruses. With the increasng prevalence of HIV infection throughout the world and especially in India, the management of opportunistic infections in patients with HIV is becoming increasingly important. The management of these opportunistic infections further depends on the accurate diagnosis of infection.

Table –16.1 Viral infections seen in HIV/AIDS

Virus	Lesions
Cytomeglovirus	Retinitis, colitis, disseminated oesophagitis, pneumonitis
Herpes simplex virus	Cutaneous or oropharyngeal ulcers, proctitis, oesophageal ulcers
Varicella zoster virus	Multidermatomal severe varicella, pneumonia, disseminated cutaneous eruptions, retinitis, hepatitis
Molluscum contagiosum	Skin–bared area
Epsterin–Barr virus	Oral hairy leukoplakia, B cell lymphoma
Papilloma viruses	Cutaneous warts, cervical dysplasia, carcinoma of cervix or anus
JC virus	Progressive multifocal encephalopathy
Parvovirus B 19	Chronic uraemia
Measles	Severe measles
Vaccinia	Severe cutaneous infection, dissemination

The type of opportunistic infections seen in HIV patients also vary with the geographic location and specific behaviour of the patients. For example Mycobacterium tuberculosis infection is more common in developing countries whereas infection with Mycobacterium avium intracellularae complex is more common in developed countries. Similarly homosexual men are more likely to develop Kaposi's sarcoma or ulcerative perirectal Herpes simplex virus infection.

16.2 Herpes viruses

The herpes viruses are a major cause of morbidity and mortality in patients with HIV/AIDS infection. Patients with advanced HIV infection and AIDS are particularly prone to severe and progressive herpes virus infections and infections are much more severe and may be life threatening. Herpes viruses may also contribute to the progression of HIV disease as cofactors, though the exact mechanism for this is not konwn in immunocompromised individuals.

The course of infection with herpes viruses can be divided into 3 phases.

(i) **Primary infection :** In which the host gets the infection for the first time, viral replication occurs leading to death of cells and is controlled by the host's immune system.

(ii) **Latent infection :** Virus genome remains present in the host's cells in an inactive form

(iii) **Reactivation :** The latent viral genome becomes active resulting in viral replication when the host's immune system is impaired.

16.2.1 Cytomegalovirus (CMV)

CMV infection is usually seen late in the course of HIV infection and is one of the most important and frequent cause of severe disease in these patients. Primary infection with CMV can be acquired at any time throughout life and most of the adults in India have acquired CMV infections as is evident by the presence of CMV antibodies. Thirty to forty percent of homosexual men shed CMV in semen or urine irrespective of their HIV status. Of those infected, about 95% have elevated IgM titres to CMV at one time or another suggesting active infection. Majority of CMV infections seen in AIDS patients are due to reactivation of latent infection by endogenous strains but super infection by additional strains from repeated exposures can also produce active CMV infection. In asymptomatic HIV infected individuals, there is usually no viraemia whereas in progressive AIDS related complex (ARC) and AIDS patients viraemia may be present.

Clinical features

CMV retinits and CMV infections of the gastrointestinal tract such as oesophagitis, enteritis and colitis are the most important presentations. CMV pneumonitis, though can occur, is not a common manifestation in AIDS patients. CMV retinits is often seen in AIDS patients with CD4 lymphocyte count of 50/mm^3 or less. In HIV infection CMV can also present with neurological complications but most often the central nervous system syndrome is due to HIV itself. The role of CMV in AIDS dementia complex is probably secondary. Endocraniopathies and glandular involvement can occur in AIDS patients with active CMV infection. CMV can also cause acute pancreatitis in AIDS patients.

Laboratory diagnosis

The diagnosis of CMV infections requires laboratory confirmation and cannot be made on clinical grounds alone.

Virus isolation

CMV can ony be cultured in human fibroblast cells (Fig. 16.1). The conventional method for culture takes 1–4 weeks for viral isolation, but by the use of centrifugation enchanced cultures, the time has been shortened to 48 hours. In this method the clinical specimens are centrifuged at a low speed on coverslips containing monolayers of human fibroblast cells. The shell vials are incubated for 24–48 hours, the coverslips are then removed and the presence of CMV is detected by the demonstration of CMV antigen by immunofluorescence using CMV specific monoclonal antibodies (Fig. 16.6) available commercially. CMV is usually isolated from urine, throat swab, buffy coat, or other tissues obtained on biopsy or postmortem examination. However, the isolation of virus from throat, urine or even blood of patients with HIV requires careful clinical interpretation as these patients may be chronic carriers for years. The isolation of virus is not feasible in most of the laboratories.

Detection of CMV antigen

The detection of CMV antigenaemia in circulating neutrophils is a sensitive and clinically useful method of detecting viraemia. Monoclonal antibodies against a CMV matrix protein pp65 are usually used. The antigen can be detected either by ELISA or immunoflourescence. The antigenemia test is usually positive prior to onset of symptoms or detection of virus by cell culture. Kits are available commercially though, they are expensive.

Serology

The presence of CMV IgM antibody is useful but not a reliable indicator of an acute infection. IgM antibodies may not be present during an active infection (false negative) or may persist for such a long time that it may not be diagnostic (false positive). Kits are available commercially.

Polymerase chain reaction (PCR)

PCR using primers from a part of genome coding for immediate early antigen have been used but this method is over sensitive. RT-PCR for CMV RNA or quantitative PCR to determine CMV load are more useful to detect active infection or to monitor antiviral therapy. Among several studies on samples from immunocompromised patients, PCR was positive more than twice as compared to culture. PCR can also detect viraemia before the onset of symptoms in AIDS patients. PCR revealed CMV DNA in CSF of almost all AIDS patients with CNS disease. The enhanced sensitivity of PCR presents some diagnostic problems in clinical settings. Sometimes it is difficult to differentiate between a previous or active infection by PCR. PCR can detect CMV DNA in leucocytes of high proportion of AIDS cases, but results do not correlate well with clinical evidence of CMV infection.

At the district level CMV antigen detection can be done using commercially available kits. Virus isolation and PCR can be done by State/National Laboratories.

16.2.2 Herpes simplex virus (HSV)

Severe HSV infections are a prominent features in AIDS patients. Most of the infections result from reactivation of latent virus. Serological studies have found that more than 95% of homosexual men who suffer from AIDS have history of HSV infection. Genital ulcerative disease due to HSV is also an important risk factor for the acquisition of HIV infection.

Clinical feature

Genital and oral infections with more extensive tissue damage account for majority of lesions due to HSV in HIV infected individuals. Progressive HSV perianal ulcers, proctitis, colitis, oesophagitis, oral lesions, pneumonia and a variety of neurologic disorders have been observed in AIDS patients.

Laboratory diagnosis

Virus isolation can be done in tissue culture cells like Vero, rabbit kidney or human diploid cells. Cytopathic effects usually appears in 24–48 hours in the form of rounding clumping and ballooning of cells (Fig. 16.2). Multinucleate giant cells, may be observed particularly with HSV-2 isolates. Virus can be easily isolated from tissues, swabs, or CSF. Monoclonal antibodies can be used to identify and type the HSV isolates by indirect immunofluorescence as described for CMV.

Electron microscopy

Presence of HSV can be rapidly determined by electron microscopic examination of negatively stained vesicle fluid.

Direct demonstration

For a rapid diagnosis of skin or mucous membrane lesion scrapings can be stained with Giemsa/PAP and the presence of multinucleate giant cells indicate infection with HSV or Varicella zoster virus (Fig. 16.3). HSV antigens can be detected in thsee smears by immunoflourescence technique using monoconal antibodies (Fig. 16.8). HSV may be detected in infected fixed cells by immunoperoxidase staining also.

Serology

May be helpful in diagnosing primary HSV infection but is of no value in recurrent infections. HSV-specific IgM can be detected in primary infection but may also be found during recurrence.

Polymerse chain reaction (PCR)

PCR is useful in detection of HSV DNA especially in CSF samples. Rapid assays may combine amplification of HSV in culture (shell vial method) with immunology or DNA detection technique like liquid or in situ hybridization.

16.2.3 Varicella zoster virus

Varicella or Herpes zoster is recognized as a frequent infection in HIV infected occuring in 8-11% of patients. In most individuals with HIV disease, Herpes zoster is a self limiting infection but in patients in an advanced stage of HIV disease, severe complications can arise. There can be disseminated lesions of lungs, liver or nervous system leading to pneumonitis, hepatitis, VZV retinitis and encephalitis. In addition it has been observed that the course of Herpes zoster is more prolonged in HIV infected individuals and there is an increased risk of developing scarring and post herpetic neuralgia.

16.2.4 Chicken pox

The illness lasts about 5 days in healthy children and it is self limited. For the normal child, mortality is less than 2 per 100,000 cases. The risk increases by over 15 fold for adults. In immunocompromised children, particulary those with leukaemia, the lesions are numerous often with a haemorrhage. Healing takes nearly 3 times longer in these children and they are at a greater risk for visceral complications which occur in 30-50% of cases and can be fatal in as many as 15% of cases.

Laboratory diganosis

Diganosis of Herpes zoster can usually be made clinically. In a small number of cases, disseminated vesicular lesions can be confused with lesions caused by HSV. An examination of the smear from the base of vesicle can demonstrate multinucleated giant cells. Direct fluorescent staining of smear using commercially available Mab can be done for demonstration of viral antigens (Fig. 16.10). In case of systemic involvement diagnosis can be established by isolation of virus in human fibroblast cells. In research laboratories PCR is being evaluated as a diagnostic tool.

16.2.5 Epstein barr virus (EBV)

A variety of syndromes associated with uncontrolled progrssion of infection occur in individuals with congenital or acquired inability to mount an adequate immune response to EBV. In young children EBV infections are asymptomatic or very mild but become severe in case of immunodeficiency. In patients with AIDS, EBV related lymphoproliferative syndrome has been observed. EBV genome is present in one third of B cell lymphomas arising in patients with AIDS and is detected in virtually all central nervous systems (CNS) lymphomas arising in these patients.

Oral hairy cell leukoplakia is a white lesion of oral mucosa that is unique to HIV infection and appears to be caused by replication of EBV in the epithelium of keratinized cells.

Laboratory diagnosis

Serology

The EBV specific antibodies are more relaible indicator of infection using EIAs. IgM antibody against EBV capsid antigen VCA develops to high titer early in the illness and declines rapidly over the next 3 months and hence a good index of primary infection. However, since IgM assays tend to be false positive and false negative, the trend is toward using a panel of genetically cloned EBV antigens to screen for IgG antibodies. Antibodies against the early antigen EA-D are diagnostic of acute, reactivated or chronic active infection as they decline rapidly and are not detectable in asymptomatic carriers. IgG antibodies against VCA is the most convenient measure of past infection.

Virus isolation

Isolation of virus is rarely used as a diagnostic procedure since no known cell line is fully permissive for EBV. The only method of growing EBV *in vitro* is to inoculate infected oropharyngeal secretions or peripheral blood leukocytes onto umbilical cord lymphocytes and to demonstrate the immortalization of the latter to produce a lymphoblastoid cell line that can be stained successfully with fluoresceinated monoclonal antibody against EBNA.

Polymerase chain reaction

Multiple polymerase chain reaction may be useful in detecting EBV, CMV, HSV and VZV in a single tube assay from cerebrospinal fluid of AIDS patients. However, the problem with virus isolation or use of PCR to detect EBV DNA in infected cells is that a high proportion of asymptomatic individuals carry the latent virus or shed the virus for life.

16.2.6 Human herpes viruses 6–8

Human herpes virus 6 has been associated with central nervous system complications like febrile seizures, encephalitis, meningitis and multiple sclerosis. These manifestations have been reported in both immunocompetent as well as immunocompromised individuals. After primary infection, HHV 6 persists in latent form and can be reactivated in immunocompromised subjects. The most reliable source of HHV 6 is saliva. PBMCs carry the virus but often in a latent or low replicative state. Only during conditon of immunosuppression like AIDS does the virus (mostly varient A) appears to be reactivated in blood. Clinically HHV 6 B has been recognized as the cause of exanthem subitum in infant and other febrile illnesses in young children. Infection with HHV 6 B variant is seen in retinal disorders in HIV infected patients. HHV 6 has also been found in the brain, particularly in the oligodendrocytes of children with HIV related encephalitis. The potential interaction of HHV 6 with other viruses has been suggested by cell culture studies. HHV 6 activates other herpes viruses like EBV, CMV and human papilloma virus and has either enhancing or suppressing action on HIV replication. One possible effect of HHV 6 on HIV infection is that it can induce the CD4 molecule on mature CD8 lymphocytes and natural killer cells, which makes these cells potentially susceptible to HIV.

Human herpes virus –8

Human herpes virus 8 is non pathogenic in majority of healthy individuals but highly oncogenic in HIV-1 infection and iatrogenic immunsupression. It is involved in the pathogenesis of Kaposi's sarcoma, primary effusion lymphoma and some cases of multicentre Castleman's disease.

Laboratory diganosis

HHV –6

HHV 6 can be dignosed in the laboratory by detection of anti HHV 6 IgG or IgM antibodies. Serological studies can be done with virus infected cells using an indirect immunofluorescent assay or ELISA available commercially. Virus can be isolated from PBMCs of patients with AIDS by co-culture with cord blood mononuclear cells. Viral load may be determined by quantitative PCR.

HHV –8

The availability of B cell lines infected by HHV 8 and not EBV permit serological studies in diverse populations. These asays have revealed strong corelation between HHV 8 and the presence of Kaposi's sarcoma or B cell lymphomas of the abdominal cavity.

16.3 Papilloma viruses

Severe disease with HPV is frequent in patients with HIV infection. The clinical range includes anogenital warts, CIN (*càrcinoma in situ*) in women; intraepthelial neoplasiaa and cancer in homosexual men. Although presence of invasive cervical cancer is part of 1993 CDC case definition of acquired immunodeficiency syndrome, there is yet no conclusive evidence to document a rise in incidence of cervical carcinoma in HIV infected patients.

Laboratory diagnosis

The diagnosis of warts is usually made clinically. As human papilloma virus cannot be cultivated, various techniques for detection and typing of HPV DNA exist, including nucleic acid hybridization and PCR. Sensitivity was best with PCR (100%) followed by dot blot hybridization. (50%) and Southern blot hybridization (38%). These tests can only be done in National Centres.

16.4 Polyoma viruses

JC and BK viruses are the commonly known human polyoma viruses. Progressive multifocal leukoencephalopathy (PML) is seen in AIDS patients. PML is an AIDS defining disease according to CDC case definition and may be the initial presentation of HIV infection. PML lesions in AIDS demonstrate increased number of JCV infected cells and extensive necrosis.

Laboratory diagnosis

PCR can detect BK virus and JCV sequences in urine of a significant proportion of HIV infected patients. The exact significance of detection of polyomavirus nucleic acid is not yet established. The techniques of fluorescent antibody staining, EM, agglutination technique, immunocytochemistry and *in situ* hybridization have demonstrated JCV infection in PML lesions.

16.5 Molluscum contagiosum

A cutaneous pox virus infection, seen more often in HIV infected persons. In 72 adult patients with AIDS, 14% were found to have Molluscum contagiosum (Fig. 16.5) with predilection for trunk and face.

16.6 Adenovirus

Adenoviruses can cause life threatening infection in imunocompromised patients ranging from pneumonia to meningoencephalitis. Adenovirus 35 has been frequently recovered from the urine of AIDS patients. Adenovirus serotypes 42–47 are exclusively isolated from immunocompro-mised patients.

Laboratory diagnosis

Diagnosis can be established by virus isolation in tissue culture in Hep2 or Hela cells. A new PCR method using primers from hexon gene is being evaluated. Serological diagnosis involves the demonstration of four fold rise in antibodies by complement fixation test, neutralization test, haemagglutination inhibition (HI) test or ELISA.

16.7 Measles virus

Severe measles may occur in patients with AIDS. In a study the case fatality rate of measles in 11 HIV infected patients was calculated to be 40%. In HIV infected patients 27% had no rash and 82% had giant cell pneumonia.

Laboratory diagnosis

Virus isolation

The virus can be easily isolated from nasopharyngeal aspirates or washings in Vero, CV-1, BSC-1 or HeLa cells and identified by syncytia or giant cell formation.

Serology

Serological diagnosis is established either by HI, neutralization, enzyme immunoassay or by demonstration of measles antigen by immunofluorescence. IFAs and EIAs have the ability to measure measles specific IgM and IgG response.

16.8 Respiratory syncytial virus

In children with HIV infection, RSV appears primarily to cause pneumonia rather than bronchiolitis and there is prolonged shedding of virus. Although the impact of RSV infection in immunocompromised patients has been recognised to be very severe and associated with high mortality.

Laboratory diagnosis

The evaluation of laboratory diagnosis methods for these high risk patients has not been systematically examined. Serological assay and virus isolation are common techniques and specimens such as nasopharyngeal swabs, nasopharyngeal washes, bronchioalveolar lavage, endotracheal tube aspirates, lung biopsies are generally used. Viral culture in cell lines as MDCK, LLC-MK2, Hep2 and WI-38 are carried out for 2 weeks. Centrifugation enhanced cultures yield more rapid results. The virus is identified by characteristic cytopathic effects and is confirmed by indirect immunofluorescence using poly and monoclonal antibodies and ELISA (available commercially).

16.9 Hepatitis viruses

Defects in cell mediated immunity in AIDS result in a wide variety of hepatic complications including granulomas, cytomegalovirus hepatitis, multimicrobial AIDS cholangiopathy, Kaposi's sarcoma and lymphoma. Kupffer cells are the major hepatic target cell population for HIV. The majority of patients with established AIDS reveal abnormalities in serum liver tests. In these patients the differential diagnosis includes opportunistic infections and neoplasms as well as chronic viral hepatitis due to viruses B,C,D and G and drug hepatotoxicity. Among these chronic viral hepatitis due to hepatitis C virus is frequently seen. It presents a more accelerated course in HIV infected patients leading to cirrhosis and liver failure in a shorter period of time. Decompensated liver disease like encephalopathy, ascitis and jaundice or complictions like gastrointestinal bleeding, hepatorenal syndrome and peritonitis are commonly diagnosed.

FIG. 16.1 HUMAN DIPLOID FIRBOBLAST CELLS INFECTED WITH CMV

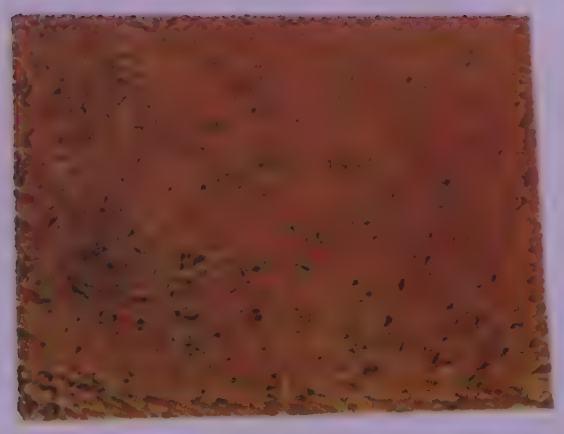


FIG. 16.2 HUMAN DIPLOID LUNG FIBROBLAST CELLS INFECTED WITH HSV

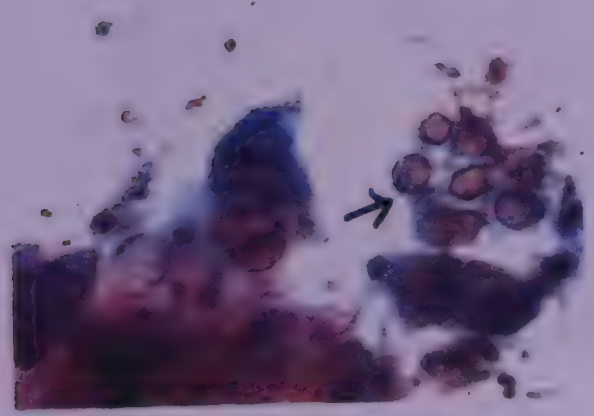


FIG. 16.3 PAP STAINED SMEAR SHOWING MULTINUCLEATE GIANT CELLS (HSV/V2V)

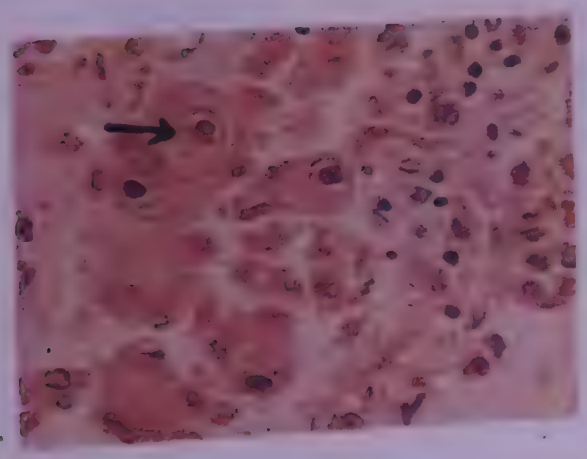


FIG. 16.4 H&E LUNG TISSUE SHOWING INTRANUCLEAR CMV INCLUSIONS WITHIN ENLARGED CELLS

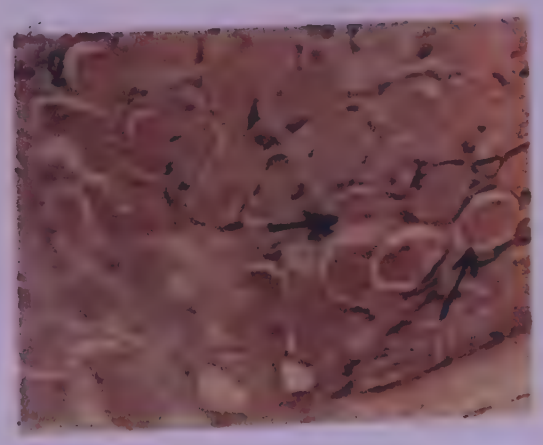


FIG. 16.5 H&E EPIDERMIS FILLED WITH MOLLUSCUM CONTAGIOSUM BODIES

FIG. 16.6 CYTOMEGALOVIRUS

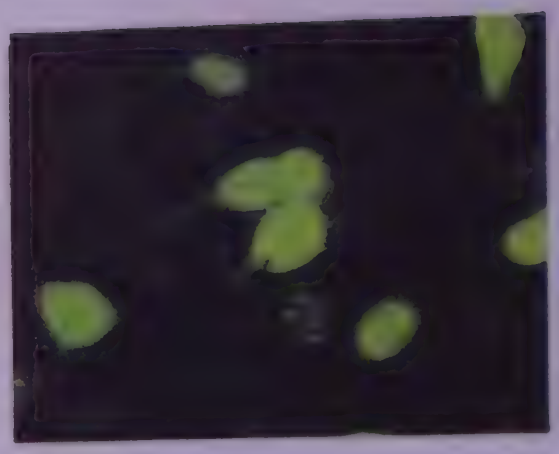


FIG. 16.7 ADENOVIRUS

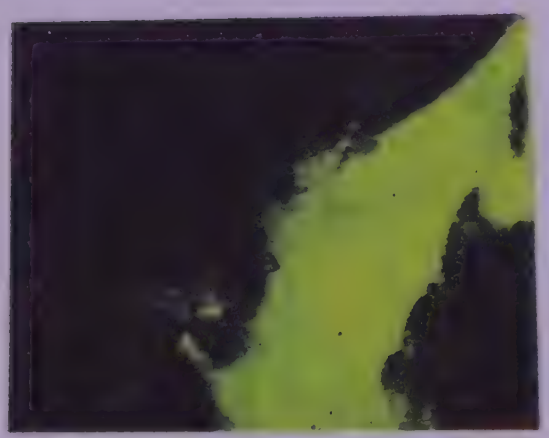


FIG. 16.8 HERPES VIRUS

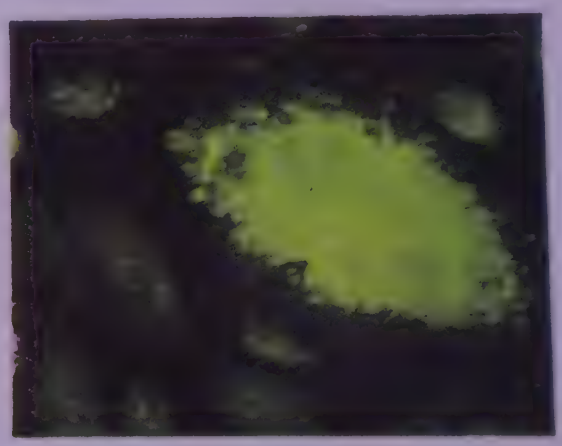


FIG. 16.9 MEASLES VIRUS

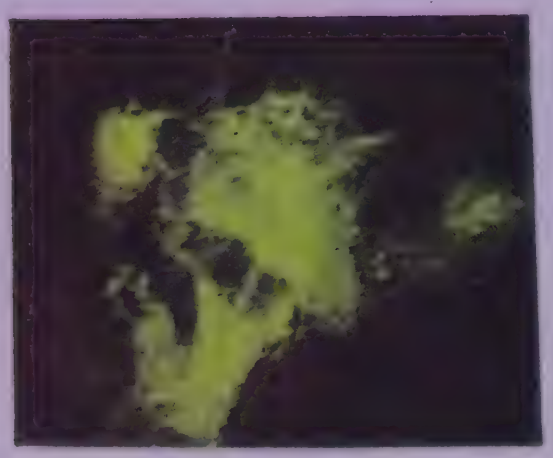


FIG . 16.10 VARICELLA ZOSTER VIRUS

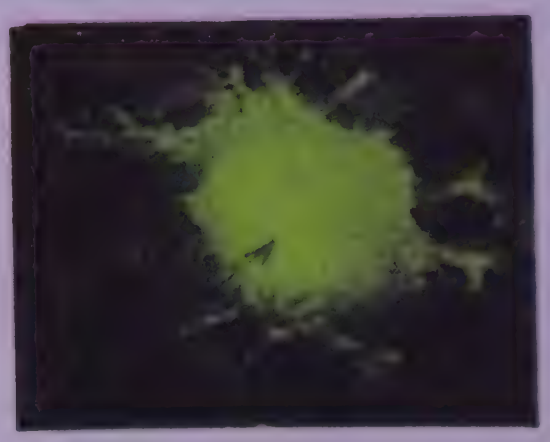


FIG. 16.11 RESPIRATORY SYNCYTIAL VIRUS

FLUORESCENT ANTIBODY STAINING OF VIRUS INFECTED CELLS (IN-VITRO CULTURE)

16.10 References

1. Masur H. 1995. Management of opportunistic infections associated with HIV infection. Principles and practice of infectious diseases. volume 2 : pp 1290 - 1293. Fourth edition. Ed. Mandell GL, Bennett JE, Dolin R. Churchill Livingstone.
2. White DO, Fenner FJ. Medical Virology 1994; pp 136–146. Fourth edition. Academic press.
3. Lennette EH, Lennette DA, Lennette ET. 1995. Diagnostic procedures for viral, rickettsial, and chlamydial infections. Sevnth edition. American public health associated.
4. Kimberlin DW, Wshitley RJ. 1998. Human herpesvirus -6 : neurologic implications of a newly-described viral pathogen. J Neurovirol; 4(5) : 474–85.
5. Lefkowitch JH. 1997. The liver in AIDS. Semin Liver Dis; 17(4) : 335–44.

Appendix 16.1

Diagnosis of CMV infections

Collection and preparation of specimens

CMV can be isolated from blood, tisues, urine, saliva tears, semen, cervical or vaginal secretions, aqueous humour and bronchoalveolar fluid.

All samples should be collected aseptically and held at 4^0 C and transported on ice. Clean catch midstream urine is collected in sterile containers and treated with antibiotics (Gentamycin 20 ìg/ml and nystain 20 U/ml). Swab or secreations are collected using sterile decron tipped applicator. After collecting the secretions the swab is placed in viral transport medium. CMV in peripheral blood is associated with polymorphonuclear leucocytes. Leucocytes can be separted from blood by density gradient centrifugation.

Method of separation of leucocytes from blood

The whole blood is settled over 6% dextran for 45 min. The leucocytes are than separated from the buffy coat by centrifugation at 800x g for 10 min. washed twice and resuspended in tissue culture media at a concentration of $5x10^6$ cells/ml.

Isolation of CMV

Specimens are collected and prepared as for conventional cell culture. Human fibroblast ($5x10^4$) cells in growth media are seeded onto 12 mm coverslips in 1-dram shell vials closed with rubber stopper and incubated for 2-4 days. Prior to inoculation of sample, medium is removed and 0.3 ml of sample per vial is inoculated and the vials are centrifuged at 1500x g for 45 min at 37^0 C. After centrifugation the inoculum is replaced with 1.5 ml of maintenance medium and the vials are incubated for 16-24 hours at 37^0 C. After 16-24 hours the coverslips are removed, washed twice with PBS and fixed with chilled acetone for 10 min. A mixture of monoclonal antibodies to immediate early antigen (IE) and early antigen are added and the coverslips are incubated for 30 mın at 37^0 C. The monoclonal antibody is removed by washing the coverslips twice with PBS and an anti-mouse FITC conjugatd antibody is added at appropriate dilution. The coverslips are incubated for 30 min at 37^0 C and then washed with distilled water twice. Counter staining is done with 0.3 ml of 2% Evan's blue. The coverslips are again washed with distilled water and mounted cell-side down on a microscope slide and examined with a fluorescent microscope. The sample is considered positive if at least one intact cell shows typical intranuclear fluorescence.

Detection of CMV antigens

Leucocyte antigenaemia

Direct detection of CMV in peripheral blood leukocytes using IFA technique with monoclonal antibodies to CMV is a rapid method for detecting viremia in immunocompromised hosts. The polymorphonuclear leukocytes are separated from peripheral blood with dextran sedimentation as described above. The cells are than cyto-centrifuged on microscope slides so that each preparation has 50,000 cells/preparation. Cell are fixed with acetone, methanol or paraformaldeldye. The cells are then treated with monoclonal antibody to CMV matrix protein pp65 for min at 37^0 C. The cells are washed twice with PBS and antimouse FITC conjugated antibody is added at optimal dilution. The cells are then washed, counter stained with Evan's blue and examined under fluorescent microscope as described above.

Annexure

TREATMENT OF COMMON AIDS ASSOCIATED INFECTIONS

Drug treatment for AIDS associated infections

The standard drugs given for management of HIV/AIDS associated infections along with the alternatives available and dose schedules are tabulated below :

Condition	Standard treatment		Alternative treatment	
	Drug	Dosage	Drug	Dosage
Tuberculosis	Isoniazid + Rifampin + Pyrazinamide + Ethambutol or Streptomycin	300 mg po daily 600 mg po daily 15–25 mg/kg po daily 15–25 mg/kg po daily 15 mg/kg IM daily		
Disseminated *M.avium* complex	Clarithromycin or Azithromycin + one or more of the following : Ethambutol Clofazimine Ciprofloxacin Rifabutin	500 mg po bid 500 mg po daily 15–25 mg/kg daily 100–200 mg daily 750 mg bid 300–450 mg po daily		
Syphilis Primary, secondary, latent	Benzathine Pencillin or Doxycycline or Erythromycin	2.4 million units IM 100 mg po bid x 14 days 500 mg po qid x 14 days	For all stages : Amoxicillin + Probenecid or Doxycycline or Ceftriaxone or Benzathine PCN + Doxycycline	 2 g po tid x 14 days 500 mg po tid x 14 days 200 mg po bid x 21 days 1 g IM daily x 5–14 days 2.4 million units IM weekly x 3 doses 200 mg po bid x 21 days
Late latent	Benzathine PCN or Doxycycline	2.4 million unit IM weekly x 3 100 mg po bid x 28 days		
Neurosyphillis	Aqueous PCN G or Procaine PCN + Probenecid	12–24 million units/day IV x 10–14 days 2.4 million units IM daily x 10 days 500 mg po bid x 10 days		
P.*carinii* pneumonia	TMP-SMX ± Prednisone	15 mg/kg/day po or IV in 3 to 4 doses x 21 days 40 mg po bid, days 1–5 20 mg po bid, days 6–10 20 mg po daily, days 11–21	Pentamidine Trimetrexate + Folinic acid Dapsone + Trimethoprim Atovaquone suspension Primaquine + Clindamycin	3–4 mg/kg IV daily x 21 days 45 mg/m² IV daily x 21 days 20 mg/m² po or IV q6h x 21 days 100 mg po daily x 21 days 5 mg/kg po tid x 21 days 750 mg po bid x 21 days 15 mg base po daily x 21 days 600 mg IV qid x 21 days, or 300–450 mg po qid x 21 days

Condition	Standard treatment		Alternative treatment	
	Drug	Dosage	Drug	Dosage
Toxoplasmosis	Pyrimethamine + Sulfadiazine	50–100 mg po daily 1–1.5 g po qid	Pyrimethamine + Clindamycin	50–100 mg po daily 450–600 mg po or 600–1200 mg IV qid
Chronic suppressive therapy	Pyrimethamine ± Sulfadiazine	25–50 mg po daily 500 mg to 1 g po qid	Pyrimethamine + Clindamycin	50 mg po daily 300 mg po qid
Cryptosporidiosis	Paromomycin	500–750 mg po qid		
Candidiais–oral	Nystatin solution or tablets	500,000 to 1,000,000 units 3–5 x/day	Fluconazole Itraconazole Ketoconazole or Clotrimazole	100–200 mg po daily 200 mg po daily 200 mg po daily 10 mg po 5 x/day
Oesophageal	Fluconazole	100–200 mg po daily x 1–3 weeks	Itraconazole Ketoconazole Amphotericin B	200 mg po daily 200 – 400 mg po daily x 2–3 weeks 0.3 mg/kg IV daily x 7 days
Coccidioidomycosis	Amphotericin B	0.5–1 mg/kg IV daily	Fluconazole	400–800 mg po daily
Chronic suppressive therapy	Amphotericin B	1 mg/kg weekly	Itraconazole Flucoazole	400 mg po daily 200 mg po bid
Cryptococcosis	Amphotericin B	0.3–1 mg/kg IV daily	Fluconazole	400–800 mg po daily
Chronic suppersive therapy	Fluconazole	200 mg po daily	Amphotericin B	0.5–1 mg/kg IV weekly
Histoplasmosis Chronic suppressive therapy	Amphotericin B Itraconazole	0.5–0.6 mg/kg IV daily 200 mg po bid	Itraconazole Amphotericin B	200 mg po bid 0.5–0.8 mg/kg IV weekly
Isosporiasis	Trimethoprim – Sulphamethoxazole	One double strength tablet twice daily		
Cyclosporiasis	–do–	–do–		
Microsporidiosis	Probably albendazole			
Cytomegalovirus Retinitis, Colitis, Esophagitis	Ganciclovir	5 mg/kg IV q12h x 14–21 days	Foscarnet	60 mg/kg IV q8h or 90 mg/kg IV q12h x 14–21 days
Chronic suppressive therapy	Ganciclovir	5 mg/kg IV daily or 6 mg/kg IV 5 x /week or 1 g po tid	Foscarnet	90–120 mg/kg IV daily
Herpes Simplex virus	Acyclovir	200–800 mg po 5 x /day	Foscarnet	40 mg/kg IV q8h x 21 days
Primary or recurrent, secondary, prophylaxis,	Acyclovir	400 mg po bid	Foscarnet	40 mg/kg IV daily
Varicella–zoster, primary or disseminated	Acyclovir	10 mg/kg IV q8h x 7–14 days	Foscarnet	40 mg/kg IV q8h
Dermatomal zoster	Acyclovir	800 mg po 5 x /day x 7–10 days	Famciclovir Foscarnet	500 mg po q8h x 7 days 40 mg/kg IV q8h

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